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Molecular Characterization and Disease-Related Morbidities of β-Thalassemia Major and Intermedia Patients from Sulaymaniyah/Kurdistan-Iraq

A Dissertation Submitted to the Department of Pathology and Forensic Pathology and the Post Graduate Studies Department at the College of Medicine-University of Sulaymaniyah in a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Molecular Hematopathology

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بِسْمِ اللَهِ الرَحْمنِ الرحيمْ

نَـرْفَعُ دَرَجاتٍ مَنْ نَشَاءْ وَ فَوْقَ كُلِ ذِي عِلْمٍ عَلِيمْ

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سورة يوسف: الأية 76

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I hereby declare that, the content of this dissertation is original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is the result of my own work and that all resources of materials have been duly acknowledged.

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Supervisors Declaration

We certify that this dissertation entitled "Molecular Characterization and Disease-Related Morbidities of β-Thalassemia Major and Intermedia patients from Sulaymaniyah/Kurdistan-Iraq" was prepared under our supervision in the Department of Pathology and Forensic Pathology, College of Medicine, University of Sulaymaniyah in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Hematopathology.

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Dedication

I dedicate this dissertation with love and eternal appreciation to my merciful mother, my beloved husband, and my most precious gifts, my children.

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ABSTRACT

Background: β-thalassemias are inherited hemoglobin disorders, which are the most common monogenic diseases worldwide, particularly in the Eastern Mediterranean region, including Iraq and Kurdistan Region. Globally, the estimated prevalence rate is 4.4/10,000 live births, with an estimated carrier rate of 1.5%. The disease hallmarks include imbalance in the α/β globin chain ratio, ineffective erythropoiesis, chronic hemolytic anemia, and enhanced intestinal iron absorption. The clinical severity of β-thalassemia varies widely ranging from asymptomatic to severe or even fatal entities which reflects the degree of globin chain imbalance that is determined by the nature of the underlying β-gene mutations. More than 350 disease-causing mutations have been identified, and have a geographical pattern with a racial origin. Bone disease, hepatobiliary complications, pulmonary hypertension, and multiple endocrine abnormalities are the most encountered disease-associated complications. Conventional management primarily relies on transfusion and iron-chelation therapy, as well as splenectomy in specific cases. In addition, an increased understanding of the molecular and pathophysiological mechanisms that govern the disease process lead to the development of new therapeutic approaches.

Objectives: The aim of this study was to characterize the spectrum of β-globin gene mutations in both thalassemia major and thalassemia intermedia phenotypes at Sulaymaniyah Thalassemia Center in northeastern Iraq. Additionally, the evaluation of patients' disease characteristics and different lines of management that are implemented at our center. Another objective was to determine the frequency of different disease-related morbidities and compare them in both thalassemia phenotypes. Finally, to evaluate the

impact of genotype on developing disease complications and genotype-phenotype correlation among the enrolled β-thalassemia patients.

Patients and Methods: This is a cross sectional study conducted on 242 β-thalassemia patients, including 159 thalassemia intermedia and 83 thalassemia major patients from 162 families who were registered and received treatment in the Sulaymaniyah Thalassemia Care Center. Detection of β-thalassemia mutations was done by reverse hybridization technique or direct gene sequencing. Also, the clinical parameter, disease characteristics and treatment modalities, with all the laboratory data, as well as, Dual Energy X-ray Absorptiometry scan for bone mineral density assessment and echocardiography for identification of pulmonary hypertension were all collected through an electronic-based medical recording system using a designed comprehensive questionnaire. In addition, full medical history and physical examination were recorded by direct interviewing the patients.

Results: A total of 22 β-globin mutations arranged in 53 different genotypes were identified, IVS II-1 (G>A) (35.7%), followed by IVS I-6 (T>C) (18.0%), and codon 8/9 (+G) (8.5%) were the most frequent. The former mutation was the most prevalent among thalassemia intermedia patients, while the later mutation was the most prevalent in thalassemia major patients. Homozygous mutations were determined in (76.3%) patients, 62.9% of which were the result of consanguinity. Among disease-related morbidities documented; bone disease was the most frequent amounted to (66.9%), followed by endocrinopathies (32.2%), hepatobiliary complications (28.9%), and pulmonary hypertension (9.9%). In contrast, venous thrombosis, and leg ulcer were less frequently observed. Lastly, using hydroxyurea therapy resulted in a potentially lower serum ferritin,

annual transfusion frequency, and chelation therapy requirement among the enrolled β-thalassemia intermedia patients.

Conclusions: The current study, the largest from Iraq and Kurdistan region on β-thalassemia patients, revealed that β^0 thalassemia mutations were the most frequent mutations in both thalassemia intermedia and thalassemia major patients and were rather distinct from reports from Iraq and nearby countries. Additionally, despite consistence with the standard management guidelines in thalassemia patients, yet complications rate is high, which encountered in 78.9% of the enrolled β-thalassemia patients. The complications were more frequent among thalassemia major patients, with an evidently higher rates in patients with $\beta^0\beta^0$, and $\beta^0\beta^+$ genotypes, with increased probability of developing complications in advanced age.

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List of Abbreviations

Abbreviation	Detail
β	Beta Globin Chain
β^{+}	Mild Beta Mutation
β^{0}	Severe Beta Mutation
5'UTR	5' Untranslated Region
ALT	Alanine Aminotransferase
ARMS	Amplification Refractory Mutation System
ASOs	Allele Specific Oligonucleotides
BCL11A	Transcription factor
BM	Bone Marrow
BMD	Bone Mineral Density
CBC	Complete Blood Count
CO	Cardiac Output
DB	Dot Blot
DEXA	Dual Energy X-ray Absorptiometry
DM	Diabetes Mellitus
DNA	Deoxy nucleic acid
Dw	Dry Weight
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme- Linked Immunosorbent Assay
EMH	Extramedullary Hemopoiesis
EPO	Erythropoietin
FPN	Ferroportin Gene

FVL	Factor V Leiden
G20210A	Prothrombin Gene Mutation
GDF	Differentiation Factor
β-globin	Beta Globin
GVHD	Graft Versus Host Disease
HAMP	Hepcidin Gene
Hb A	Adult Hemoglobin
Hb F	Fetal Hemoglobin
HBS1L	Elongation Factor
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HFE	Human Hemochromatosis Gene
HIFs	Hypoxia-Inducible Transcription Factors
HIV	Human Immune Deficiency Virus
HJV	Hemojuvelin Gene
HLA	Human Leucocyte Antigen
HPLC	High-Performance Liquid Chromatography
HSCT	Hematopoietic Stem Cell Transplantation
HU	Hydroxyurea
IU/l	International Unit/Liter
IVS	Intervening Sequence
JAK 2	Janus Activated Kinase 2

Kb	Kilobase
KLF 1	Kruppell-Like Factor 1
β-LCR	Beta-Locus Control Region
LFT	Liver Function Test
LIC	Liver Iron Concentration
LPI	labile Plasma Iron
LV	Left Ventricle
MCH	Mean Cell Hemoglobin
MCV	Mean Cell Volume
MED	Mediterranean
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MTHFR	Methyl Tetra Hydro Folate Reductase Mutation
MYB	Myeloblastosis Oncogene
NCHS	National Center for Health Statistics
NTBI	Non-Transferrin Bound Iron
NTDT	Non-Transfusion Dependent Thalassemia
O_2	Oxygen
PASP	Pulmonary Artery Systolic Pressure
PCR	Polymerase Chain Reaction
PHT	Pulmonary Hypertension
PND	Prenatal Diagnosis
PVR	Pulmonary Vascular Resistance
QTL	Quantitative Traits Locus

RBC	Red Blood Cell
RDB	Reverse Dot Blot
RDW	Red Cell Distribution Width
RE-PCR	Restriction Endonuclease-Polymerase Chain Reaction
RES	Reticuloendothelial System
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
SQUID	Superconducting Quantum Interference Device
TDT	Transfusion Dependent Thalassemia
TFR2	Transferrin Receptor 2 Gene
TGFB1	Transforming Growth Factor β1
β-thal	Beta Thalassemia
TI	Thalassemia Intermedia
TIF	Thalassemia International Federation
TM	Thalassemia Major
TMPRSS6	Metallo Protease
TSH	Thyroid Stimulating Hormone
UGTIA1	Uridine Diphosphate Glucouronosyl Transferase 1A
VDR	Vitamin D Receptor
WHO	World Health Organization
α	Alpha Globin Chain
γ	Gamma Globin Chain
δ	Delta Globin Chain

ε	Epsilon globin chain
ζ	Zeta Globin Chain

Introduction

Beta thalassemia (β-thal) is one of the most widely distributed autosomal recessive disorders which affects the β-globin gene of hemoglobin with a predominant incidence in the Mediterranean countries, North Africa, the Middle East, India, Central and Southeast Asia. It imposes a significant health burden, particularly in the under-resourced countries, including Iraq and Kurdistan [1, 2]. The underlying pathophysiology of the disease is multifactorial and stems from a reduced or absent generation of the β-globin chain of the hemoglobin causing α:β chain imbalance, intracellular accumulation of free α-chains and subsequent red cell destruction ending in ineffective erythropoiesis, chronic hemolytic anemia, compensatory hemopoietic expansion, hypercoagulability, and increased iron absorption from the gut; the hallmark of β-thalassemia [3].

Over 350 various mutations of the β-globin gene were recorded, ranging from silent mutations (silent β), to mild mutations that cause a relative reduction in β-globin chain production (β^+, $\beta^+\beta^+$), to severe mutations that result in a complete absence of β-globin chain synthesis (β^0) [4]. The broad spectrum of β-thal alleles can produce a wide spectrum of different β-thal phenotypes [5, 6]. These mutations are not uniformly distributed, but have a geographical specificity and racial origin, as each population is characterized by the presence of few common mutations and variable numbers of rare ones [7]. These β-thal mutations were reported in every step in the pathway of β-globin gene expression; transcription, messenger ribonucleic acid (mRNA) processing, mRNA translation, and post-translational integrity of the β-globin chain, and majority are due to the point mutations with deletions of the gene being uncommon [8]. There are

several molecular techniques used for the diagnosis of β-globin mutations, previously amplification refractory mutation system (ARMS) and restriction fragment length polymorphism (RFLP) were the principle techniques for diagnosis, but they are labor-intensive, slow and expensive. Therefore, modern molecular biology technique, such as reverse hybridization strip assay method have been implemented for the diagnosis of thalassemia, which are more reliable, simple, less expensive, fast and most applicable for β-thal mutation detection [9].

The clinical manifestation of β-thal are extremely diverse, at one end of the spectrum is β-thal minor (trait or carrier), a clinically silent, mildly hypochromic and microcytic anemia, although others can have no identified hematological abnormalities (silent carriers). At the other end is β-thal major (TM) which refers to those patients whose clinical course is characterized by profound anemia, present to medical attention in the first year of life, and subsequently require regular blood transfusions and iron chelation therapy for survival. The term β-thal intermedia (TI) represents patients with clinical manifestations that are too severe to be termed minor, yet, milder to be termed major, presenting later in life with mild-moderate anemia and variable transfusion requirements, although there remains substantial overlap between the three conditions [7, 10, 11]. Over the past decade, labelling of thalassemia has changed moving away from the molecular to a clinical categorization largely based on the frequency and magnitude of transfusion requirements which indirectly reflects the underlying severity of the disease. Thus, patients are categorized as transfusion dependent thalassemia (TDT), patients who are not capable of producing sufficient hemoglobin to survive without blood transfusion, or

non-transfusion dependent thalassemia (NTDT), in which patients can still require transfusion therapy sporadically, or even regularly, but not for their entire lifetime [10-12]. Management guidelines for both patients with TM and TI are available as part of the global efforts of the Thalassemia International Federation (TIF), including transfusion therapy, iron chelation therapy, splenectomy, modulation of Hb F production, and hematopoietic cell transplantation [10, 12].

Despite the significant progress and advances made in different treatment modalities in clinical practice over the past decades, yet there are multiple serious morbidities which arise from chronic anemia, and progressive iron accumulation in different organs as a consequence of repeated transfusion therapy, as well as enhanced iron absorption from the gut [13, 14]. In addition, many patients with TM, especially those living in developing countries, do not have access to conventional and/or innovative treatment approaches that are capable of reducing the accumulation of iron in body organs particularly the heart, liver and pancreas, which has dramatically improved survival rates [15, 16].

Bone disease (facial bone deformities and osteoporosis), hepatobiliary complications [abnormal liver function tests (high ALT ≥50 IU/l) and biliary (cholelithiasis and cholecystectomy)], pulmonary hypertension (PHT), and multiple endocrine abnormalities (growth retardation, hypogonadism, hypothyroidism, and diabetes mellitus) are the most encountered disease complications [17]. Furthermore a high frequency of chronic hepatitis C as well as psychosocial morbidity associated with chronic disease remains a challenge [18].

Aims of the Study

The present study was designed to:

1. Investigate the spectrum of β-globin gene mutations in both thalassemia major and thalassemia intermedia phenotypes at Sulaymaniyah Thalassemia Center in northeastern Iraq.
2. Evaluation of the demographic, clinical, hematological, and therapeutic approaches of β-thalassemia patients that were implemented at Sulaymaniyah Thalassemia Center in comparison with different studies in Iraq, neighboring and other Mediterranean countries.
3. Determine the frequency of different disease-related morbidities, and comparing the results in between both β-thalassemia phenotypes.
4. Assess genotype-phenotype correlation among our β-thalassemia patients.

CHAPTER ONE

LITERATURE REVIEW

1.1 Definition and Classification of Thalassemia

The thalassemias are a heterogenous group of genetic disorders of Hb synthesis, all of which result from absent or reduced production of one or more of the globin chains of Hb. They are divided into the α, β, δβ or γδβ thalassemias, according to which globin chain is affected. Genetically, when no globin chain is synthesized at all, thalassemia are designated as α^0 or β^0 thalassemias; others are designated α^+ or β^+ thalassemias when the globin chain is produced at a reduced rate [(4)].

Furthermore, clinically thalassemias are classified according to their severity into minor, intermedia and major forms. Thalassemia minor (trait or carrier) represents the heterozygous inheritance of α or β-thalassemia mutation, with patients often have asymptomatic microcytic anemia, although others can have no identified hematological abnormalities, so called silent carriers. Patients with thalassemia major usually present with severe anemia in infancy and become transfusion dependent for life, whereas patients with thalassemia intermedia can present later in life with mild-moderate anemia and variable transfusion requirements [(10, 11, 19)].

1.2 The β-Thalassemias

1.2.1 Epidemiology

β-thalassemia is prevalent in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as countries

along the north coast of Africa and in South America [20]. The highest carrier frequency is reported in Cyprus (14%), Sardinia (10.3%), and South-east Asia. The high gene frequency of β-thal in these regions is most likely related to the natural selection that has a protective role against *Plasmodium falciparum* malarial infection [20].

Population migration and intermarriage between different ethnic groups has introduced thalassemia in almost every country of the world, including Northern Europe where thalassemia was previously absent. It has been estimated that about 1.5% of the global population (80 to 90 million people) are carriers of β-thal, with about 60,000 symptomatic individuals born annually, the great majority being in the developing world. The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world. However, accurate data on carrier rates in many population are lacking, particularly in areas of the world known or expected to be heavily affected [21].

In the developed countries, thalassemia patients can survive for 25-55 years, depending on the patient's compliance to medical treatment. However, in developing countries, most of the affected patients die before the age of 20 years, mostly due to lack of effective treatment [22].

In Iraq, the incidence of thalassemia in 2015 was 34.5/100,000 which was higher than the global and the European estimated incidence rates [23]. Furthermore, it was also higher than the incidence rate reported in the Kingdom of Bahrain (0.3/100,000 in 2007) [24]. However, the incidence of thal in Iraq was lower than the rates reported in some neighboring countries

such as Oman (80/100,000 in 2010) [25], Egypt (66/100,000 in 2014-2015) [26], as well as lower than the rate reported in Italy (46/100,000 in 2013) [27].

A preventive program for hemoglobinopathies based on the concept of premarital screening, counselling, and prenatal diagnosis (PND) is the only viable way to reduce the birth of affected babies and decreasing the incidence to a much lower level [23].

1.2.2 Molecular Pathology of β-Thalassemia

The β-thalassemias are recessively inherited Hb disorder, in which individual inheriting one abnormal β-gene are asymptomatic (carrier state), while the inheritance of two abnormal β-genes is necessary to produce the disease and become clinically evident. The disease severity and the amount of synthesized globin protein are directly associated with the β-globin gene mutation. Today, over 350 unique β-thal mutations have been well recognized. The majority are point mutations (i.e. single-base substitution) and minor insertions or deletions of 1-2 bases within the gene complex itself or it's immediate flanking sequences, few deletions may also cause β-thalassemia [28, 29]. The distribution of these mutations differs in different part of the world and in different ethnic groups, although generally only a few (4-6) mutations are common in each particular population, reflecting natural selection due to malaria [28].

The mutation may affect any level of the genetic regulation in globin chain production. A variation of these kind of mutations will affect the time of initiation of transfusion, frequency of transfusion requirements, and clinical appearances in the patient's life. The mutations include:

1.2.2.1 Transcriptional Mutation

The mutations that affecting transcription include deletions and point mutations, involving the 5' untranslated region (5' UTR) of the β-globin gene as well as in the proximal CACC box. Moreover, the mutation can also happen in the TATA box region, majority of which down regulate the β-globin gene to a varying degree (Figure 1.1). Generally, they result in a mild to minimal reduction in β-globin output, which reflects the relatively mild phenotype of these β^+ thalassemias [30].

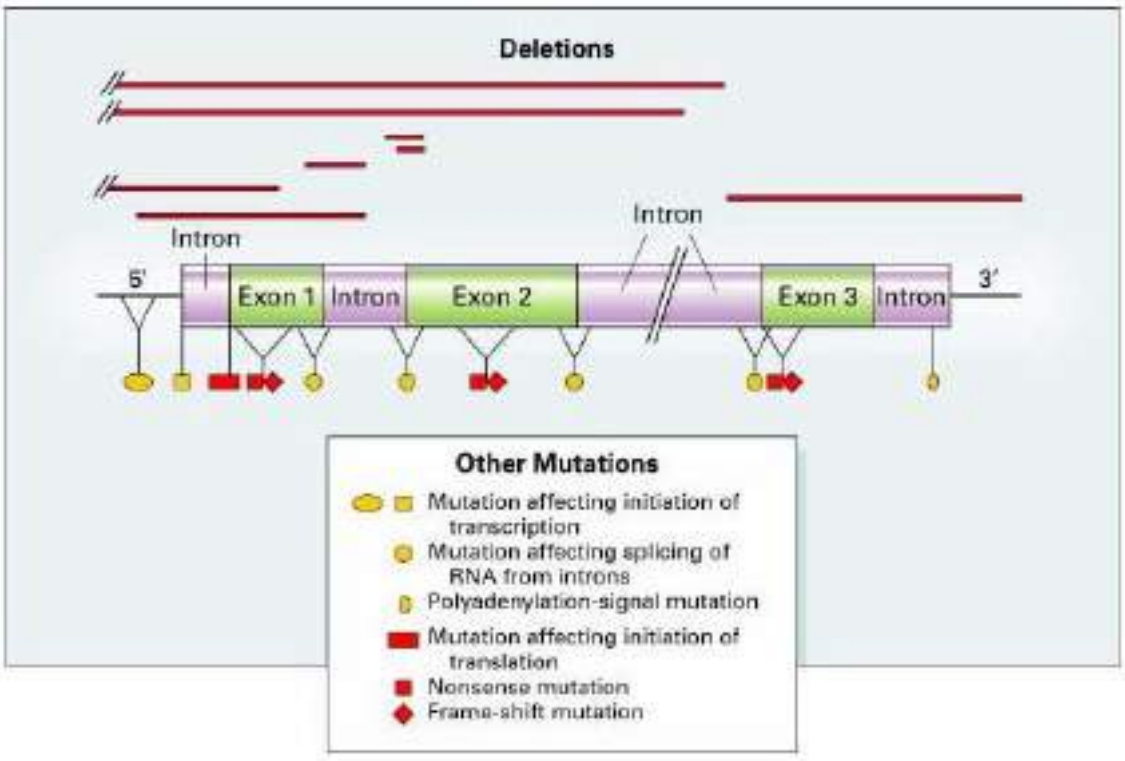

Figure 1.1: The normal structure of the β-globin gene and the locations and types of mutations resulting in β-thalassemia [31].

A couple of β-thal mutations in this group are 'silent'; the (C-T) mutation at position -101 and CAP +1 (A-C) in the 5'-UTR of the β-globin gene, which results in an extremely mild deficit of β-globin production, in which

an individual in the heterozygous state have normal red cell indices and normal Hb A_2 level. Overall, these 'silent' β-thal mutations are uncommon, except for the -101 (C-T), which constitutes a large number of the milder form of β-TI in the Mediterranean countries [32].

1.2.2.2 messenger Ribonucleic Acid (mRNA) Processing

A wide spectrum of different mutations affects the processing of the primary mRNA transcript, and this interference may involve introns, exons or their junctional sites. These mutations are of particular importance, because of the remarkable variation in their resultant phenotype. Mutations that affect either of the invariant dinucleotide (GT at 5' and AG at 3') in the splice junction cause splicing to be completely abolished and resulting β^0 phenotype. On the other hand, some other mutations at the splice junction close to the GT or AG dinucleotides in the introns reduce the efficiency of normal splicing to varying degrees and produce a β^+ phenotype that ranges from mild to severe [33]. The mRNA elongation process can be interrupted by nonsense mutation or a frameshift mutation. These mutations cause a premature termination of the mRNA and result in a severe thal phenotype (β^0). Some of the β^+ and β^{++} phenotypes are caused by a mutation in the polyadenylation region (AATAAA) at 3' position at the end of the β-globin gene that can destabilize the mRNA chain [34] (Figure 1.2).

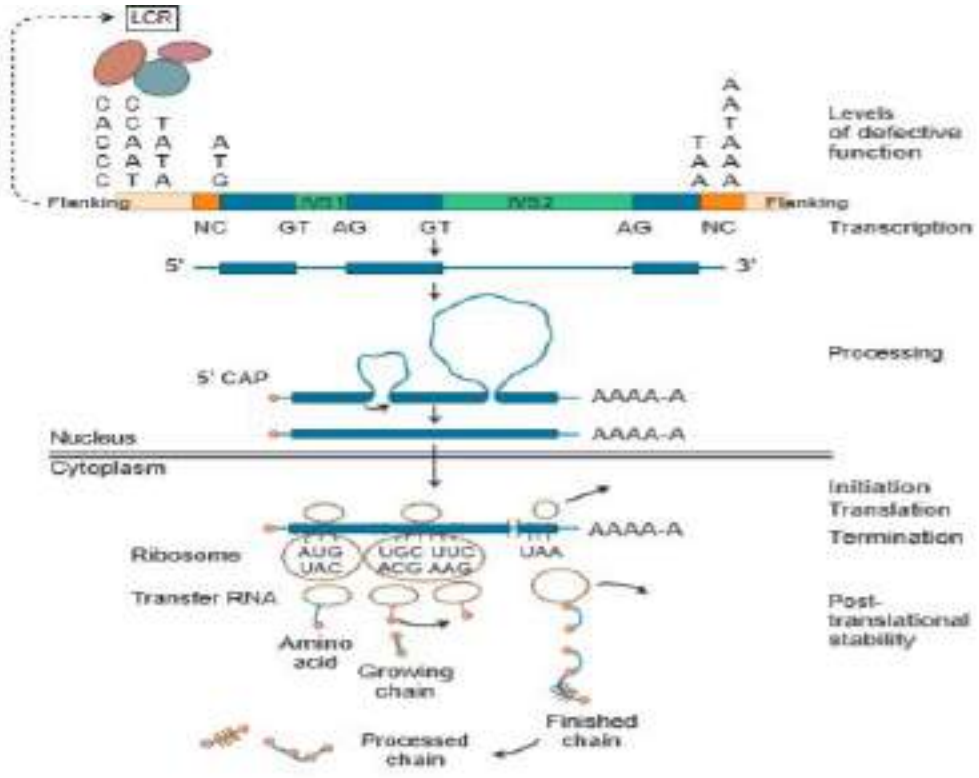

Figure 1.2: A prototype globin gene and the genetic control of globin chain synthesis with levels of action of mutations [35].

1.2.2.3 mRNA Translation

Mutations that abolish mRNA translation results in a β^0 phenotype, affecting the initiation or extension phase of globin chain synthesis. Approximately half of the β-thal mutations attributed to premature termination of the β-globin chain extension, through introduction of termination codon by either frameshift mutation that is one or more bases are lost or inserted, or nonsense mutations that is single base substitution [36].

1.2.2.4 Post Translational Stability

Some forms of β-thalassemia result from instability of the β-globin gene product, which form the basis of dominantly inherited β-thalassemias [37]. In this form of thalassemia, the inheritance of a single β-thal allele results in a clinically detectable disease in the form of TI phenotype, presented with moderate anemia, splenomegaly and a thalassemic blood picture [38]. It is characterized by the formation of large inclusion bodies in the erythroblasts, hence called 'inclusion body β-thalassemia'. It appears that some of the premature termination mutations, for example nonsense mutations that occur in exon 3 or beyond of the β-gene are not subjected to the surveillance mechanism of nonsense mediated decay and hence mutant mRNA accumulates then transported to the cytoplasm and translated. The result may be long, unstable β-globin gene products, highly unstable, non-functional and not able to form viable tetramers. Together with the redundant α-chains precipitate in the erythroid series, causing premature destruction of these cells and consequently ineffective erythropoiesis (Figure 1.3) [39].

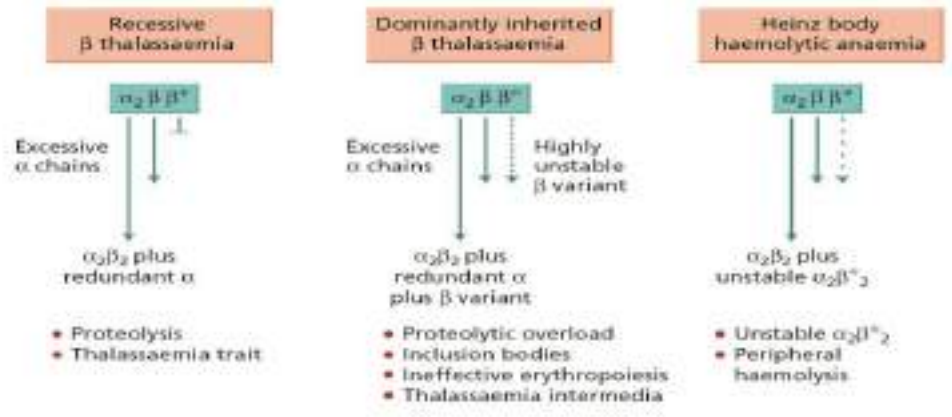

Figure 1.3: Heterozygous mutations in the β-globin gene and the different phenotypes [35].

1.2.2.5 Deletions Causing β-Thalassemia

β-thalassemia is rarely caused by deletions, the size of which varies widely, mostly removing a region in the 5'-β-globin gene promoter sequences. It results in a complete lack of β-globin product leading to a severe phenotype. It is now obvious that these rare β-thal alleles in the heterozygous state are associated with variable increase in Hb F and Hb A_2 levels, and in the homozygous state the severity of the phenotype is offset by concomitant increase in Hb F, that reflects the competition between the γ and δ gene promoters for interaction with the upstream β-locus control region (β-LCR). The unusually high Hb A_2 that accompany point mutations involving β-promoter sequence might also be explained by the later mechanism [(40)].

1.2.2.6 Unusual Causes of β-Thalassemia

These are remarkably rare but also explain several various molecular mechanisms that downregulate the β-globin gene. Transposable elements may occasionally disrupt human genes and result in their activation. The insertion of such an element, a retrotransposon of the family called L1, into intron 2 of the β-globin gene has been reported to cause β^+ thalassemia. Despite the insertion of 6-7 kb DNA into its IVS2, the affected gene expresses full length β-globin transcripts at a level corresponding to approximately 15% of normal β-globin mRNA, in which the transcript level of the affected β-globin gene was severely reduced with a decreased half-life [(41)].

Mosaicism due to somatic deletion of β-globin gene has been described causing moderately severe thalassemia intermedia in individual with

heterozygous state for β^0 (39 C-T) thal-mutation with a normal α genotype, but subsequent investigations declared that a somatic deletions of a region of chromosome 11p15 including the β globin complex had led to a mosaic of cells, 50% with one, and 50% without any normal β globin gene. The sum total of the β globin product is about 25% less than the normally asymptomatic β-thal carrier [(42)].

1.2.3 Clinical Classification

Over the past decade, a gradual transition in the labelling of the thalassemia has occurred, moving away from the molecular form to a more simplified categorization largely based on clinical-management criteria. Transfusion therapy remains the basis of management for these disorders, and the frequency and magnitude of transfusion requirements indirectly reflect the underlying severity of the disease. Thus, patients are commonly categorized as transfusion dependent thalassemia (TDT), patients who are not capable of producing sufficient hemoglobin to survive without blood transfusion, or non-transfusion dependent thalassemia (NTDT), in which patients can still require transfusion therapy sporadically, or even regularly, but not for their entire lifetime. Patients with β-TI fall under the classification of NTDT; whereas β-TM patients are classified as TDT [(10-12)].

Both TDT and NTDT are categorized based on clinical variables and a patient might move from one group to another as a result of variations and advances in clinical management, or because of changes in other disease modifiers [(10-12, 43)].

1.2.4 Pathophysiology

The underlying disease process in both β-TM and β-TI remains similar. The pathophysiological mechanism is multifactorial, and stems from reduced or absent generation of the β globin chain of the hemoglobin, causing α:β chain imbalance and subsequent ineffective erythropoiesis; the hallmark of β thalassemia [3].

Despite the significant progress and availability of different treatment modalities in the clinical practice, yet there are multiple serious morbidities which arise from ineffective erythropoiesis, chronic hemolytic anemia and progressive iron overload in different organs (Figure 1.4) [13, 14].

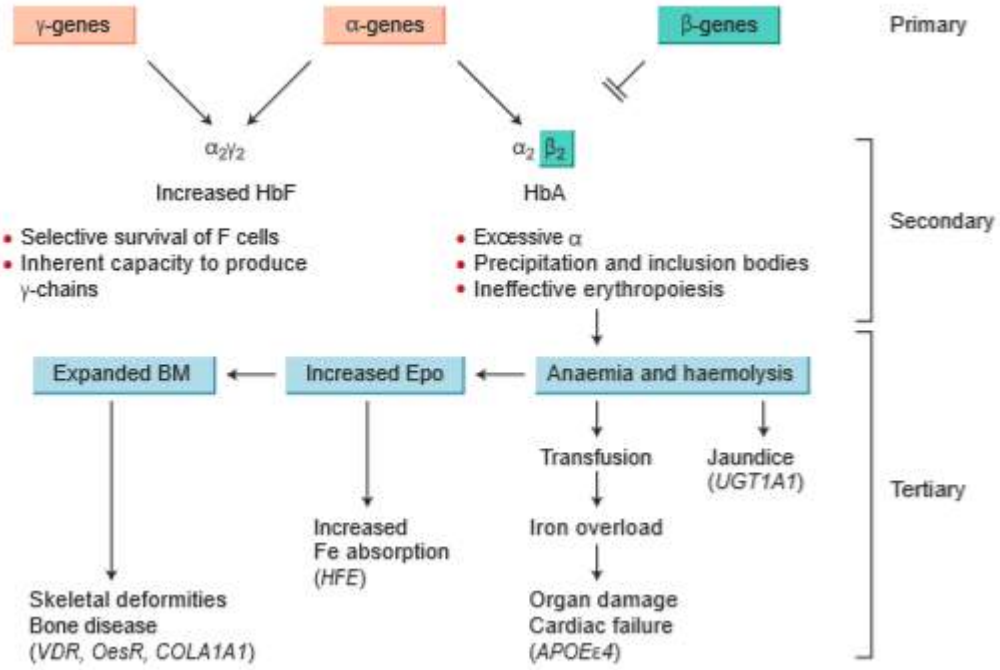

Figure 1.4: The pathophysiology of β-thalassemia [44].

1.2.4.1 Ineffective Erythropoiesis

In β-thalassemia, the free α globin chain tetramers are highly unstable and precipitate within erythroid precursors in the bone marrow (BM), causing membrane damage and premature cell death inside BM (intramedullary destruction), leading to a chronic state of anemia and ineffective erythropoiesis [(3)].

The increase in erythropoietin production driven by anemia ultimately expands the erythroid lineage within BM and lead to serious deformities of the skull and long bones, as well as compensatory extramedullary hemopoiesis (EMH) causing hepatosplenomegaly and/or pseudotumor formation, anywhere throughout the body [(45)].

1.2.4.2 Chronic Hemolytic Anemia

Both intravascular and extravascular hemolysis can occur. In TI, the higher the degree of anemia are associated with higher prevalence of complications [(46)]. The degree of ineffective erythropoiesis is the primary determinant of the severity of anemia, while peripheral hemolysis of mature red cell remains secondary [(31)].

Hemolysis mainly associated splenomegaly, and studies revealed that exposure of the erythroid cells to senescence antigens such as phosphatidylserine during ineffective erythropoiesis make a prothrombotic potentials, alongside with other factors, like chronic platelet activation, and enhanced platelet aggregation is also the hallmark of a hypercoagulability state in TI [(47)]. Furthermore, chronic hemolysis associated with chronic hypoxia, formation of reactive oxygen species and dysregulation of hepcidin/iron homeostasis, which leads to an increase in gastrointestinal

iron absorption resulting in iron overload which in turn cause several serious complications. Both anemia and iron overload can further worsen ineffective erythropoiesis and complicate the pathophysiologic picture [48].

1.2.4.3 Iron Overload

In the human body there is no active mechanism exists to excrete excess iron. The mechanism of iron overload in TM and TI is different in many aspects. In TM iron loading occurs much earlier in life and mainly as a result of lifelong obligatory blood transfusion, while in TI iron accumulation occurs much later in life and primarily due to the enhanced intestinal iron absorption, in which 2-5 g of iron accumulates per year [49].

In TI the combination of ineffective erythropoiesis [leading to increased growth and differentiation factor 15 (GDF 15)] with chronic anemia/hypoxia [altering the expression of hypoxia-inducible transcription factors (HIFs)] results in reduced expression of hepcidin; a hepatic peptide that plays a central role in iron homeostasis. Additionally, there is an increased iron absorption from the gut and increased release of recycled iron from the reticuloendothelial system (RES) (Figure 1.5). This results in depletion of macrophage iron, relatively low levels of serum ferritin, and preferential portal and hepatocyte iron loading leading to an increase of liver iron concentration (LIC) [50-53]. As a consequence, serum ferritin level underestimates the extent of iron overload in TI. The common endpoint is increases iron availability and release in the circulation with subsequent end-organ damage to the heart, liver endocrine glands, and others. Finally, an exacerbating effect of splenectomy on hemosiderosis has been suggested [54].

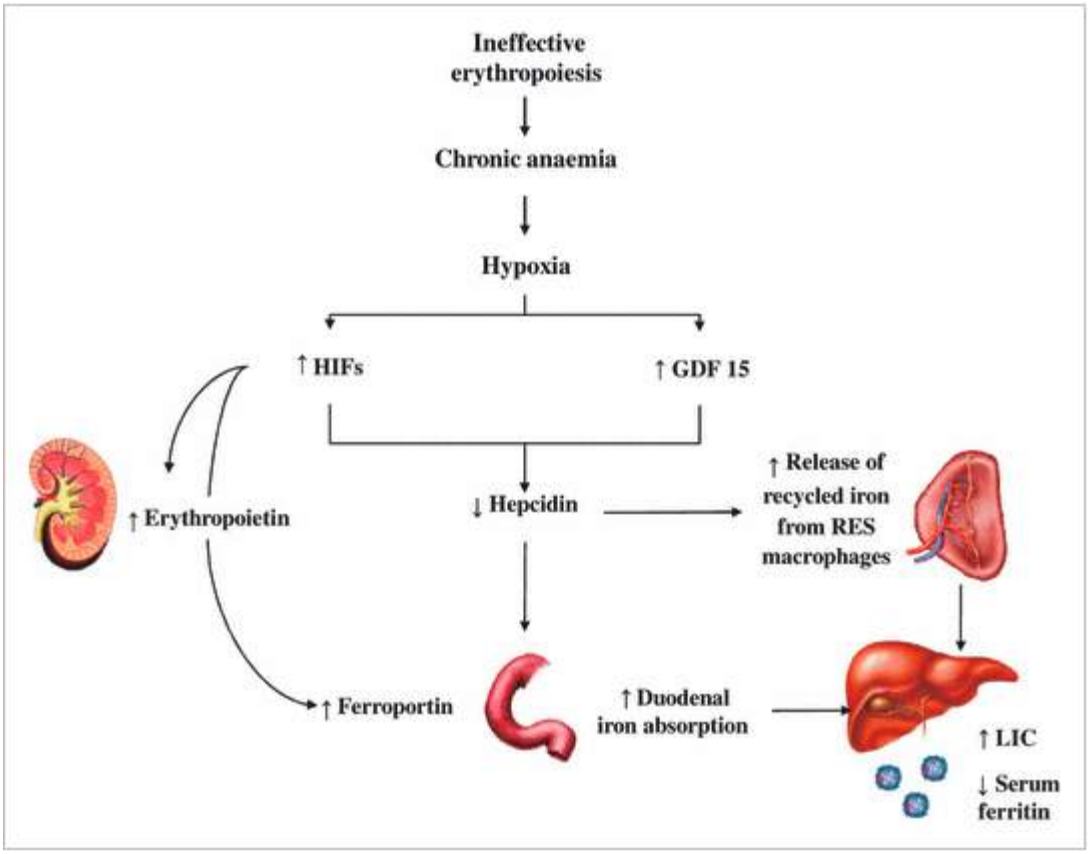

Figure 1.5: Iron metabolism in transfusion-independent patients with thalassemia intermedia [43].

1.2.5 Molecular Pathophysiology and Clinical Diversity of β-Thalassemia

Progress in our knowledge of the mechanisms underlying the extreme phenotypic variability of β-thalassemia has become possible by combination of the analysis of the different forms of thalassemia, family studies, and genotype/phenotype correlation of the thalassemia intermedia. Table 1.1 Shows common genetic interactions that underlie β-thalassemia intermedia phenotype.

Table 1.1: Molecular basis of β-thalassemia intermedia [35].

Homozygous or compound heterozygous state for □-thalassaemia
Inheritance of mild β-thalassaemia alleles (homozygous or compound heterozygotes)
Compound heterozygosity for a mild and a more severe allele
Coinheritance of α-thalassaemia
Increased HbF response
β-Globin gene promoter mutations (deletional or non-deletional)
Coinheritance of HbF quantitative trait loci
Linked: Xmn1-$^{G}\gamma$ polymorphism
Unlinked: BCL11A gene (chromosome 2p), *HBS1L-MYB* intergenic polymorphisms (chromosome 6q) *KLF1* variants
Heterozygous state for □-thalassaemia
Coinheritance of extra α-globin genes as triplicated (/ααα) or quadruplicated (/αααα) globin complexes or segmental duplication of entire α-globin cluster
Dominantly inherited β-thalassaemia (hyperunstable β-globin chain variants)
Compound heterozygosity for □-thalassaemia and □-chain variants
HbE/β-thalassaemia
Compound heterozygosity for □-thalassaemia and HPFH or □□-thalassaemia
Homozygosity for □□-thalassaemia

1.2.6 Ameliorating Factors of β-thalassemia

The ameliorating factors of β-thal acts at three levels; primary, secondary, and tertiary modifiers (Figure 1.4):

1.2.6.1 Primary Modifiers

Generally, refers to the nature of the mutation affecting the β-globin gene itself. With the exception of a few deletions, the vast majority of β-thal are caused by point mutations within the gene or its immediate flanking sequence [(55)]. These mutations have a variable effect on β-globin gene expression, which reflects the variable β-globin chains output, ranging from zero to a very minimal reduction. Location of the mutations within different gene regions determines the phenotypic severity, therefore the point mutations affecting the β-globin expression belong to three different categories: mutations leading to defective β-globin gene transcription (promoter and 5' UTR mutations), usually result in a mild deficit of β-globin production that reflects the relatively mild phenotype of these β^+ thalassemias [(7)]. Mutations affecting β-globin mRNA processing are located within 5'- and 3'-splice junctions (donor and acceptor site), as well as within splice junctions' consensus sequences. Mutations altering the donor and acceptor splice site lead to deficiency of functional mRNA production resulting in complete absence of β-globin polypeptide chains and, hence, to β^0 thalassemia. On the other hand, mutations affecting consensus sequences surrounding splice-junction, decrease the efficiency of the normal splicing to varying degrees, hence producing β-thalassemia phenotype that ranges from mild to severe. Also, these mutations could affect cryptic splice site, sequence that mimics a consensus sequence, leading to low efficiency

splicing and therefore milder form of β-thalassemia. Cap-site mutations, as well as mutations affecting polyadenylation also lead to mild β^+ thalassemia phenotype [34]. Mutations disrupting the mRNA translation either in initiation or elongation phase, result in β^0 thalassemia phenotype. Most of these defects result from the introduction of premature termination codons due to frameshift or nonsense mutations and nearly all terminate within first and second exon [56].

Functionally, β-thal alleles are classified as β^0, β^+, β^{++}, and 'silent β-allele reflecting the resulting phenotype. β^0 thal in which there is complete lack of generation of β-globin chain and the most severe form, while β^+ in which there is relative reduction in β-globin chain production. Mild β-thal, referred to as β^{++} allele with moderate amount of β-globin chain production, where the homozygous state results in intermediate phenotype, while interaction with a severe allele is less predictable due to the wide range of β-globin output, extending from transfusion dependent to intermediate forms of β-thalassemia with a milder phenotype of the spectrum. On the other hand, the uncommon silent β-thal, in which there is minimal β-globin chain deficit, and carriers have minimally reduced or normal red cell indices, and their Hb A_2 levels are normal. The 'silent' mutations are uncommon, and usually identified in patients with mild form of β-thalassemia intermedia phenotype, when combined with a severe β-thal mutation in a compound heterozygous state, or in homozygous state in individuals with typical phenotype of β-thal trait [44].

The variable severity of the different β-thalassemia alleles is reflected in their phenotypic effect in heterozygotes, in the degree of hypochromia and

microcytosis as indicated by the mean cell hemoglobin (MCH) and mean cell volume (MCV), respectively [57].

1.2.6.2 Secondary Modifiers

The clinical pictures of thalassemia that are caused by a primary mutation of the β-globin gene can be modified by a secondary genetic factor outside the β-globin gene by repairing globin chain imbalance. The secondary modifiers include coinheritance of α thalassemia, and increase Hb F response [58].

α/β Globin Ratio Modifier

It has been reported that homozygous or compound heterozygous β-thal that coinherit α-thal will have lower level of redundant α-globin and hence less severe phenotype. The degree of amelioration depends on the severity level of β-thal alleles (whether β^0 or β^+) and the number of functional α globin gene [7].

Coinheritance of single α-gene deletion has a minimum impact, while individuals with two α gene deletions and homozygous β-thal shows a less severe phenotype. In addition, patients with homozygous β-thal who coinherit Hb H disease (3 α gene deletion) will have TI [59]. In contrast, extra α-chain will aggravate the condition in β-thalassemia. Individuals with asymptomatic β-thal trait when coinherited with one or two copies of triplicated (/ααα) or quadriplicated (/αααα) α genes will convert the condition to symptomatic TI [60, 61]. Furthermore, the coinheritance of two extra α genes (ααα/ααα) or (αααα/αα) with heterozygous β thal results in TI [59].

Increasing Hb F Production

The ratio of α/β chain imbalance in β-thal can be modified by an increase Hb F production through involving genes that are included in the Quantitative Traits Locus (QTL). There are at least three main loci that are associated with the increased production of Hb F and affect the phenotype of β-thalassemia patients. Those loci are the *Xmn*I -158 C>T promoter polymorphism in the *HBG*2 gene, the BCL11A gene located on chromosome 2, and the *HBS1L-MYB* intergenic region on chromosome 6 [(62)].

About 1/3rd of the genetic variance is *Xmn*1-$^{G}\gamma$ polymorphism, but over 50% of the genetic variance in F-cell levels are contributed to factors not linked to the β-chromosome, in which some patients revealed to have enhanced Hb F response despite being *Xmn*1-$^{G}\gamma$ -/-, and linkage studies have mapped loci controlling Hb F and F-cell levels to three region of the genome; chromosome 6p23, Xp22, and 8q11 [(63-65)]. The *Xmn*1-$^{G}\gamma$ site is common and present at a frequency of 0.32-0.35 of population, and does not always raise Hb F levels in otherwise normal individuals. Clinical studies have shown that, under conditions of hematopoietic stress (e.g. homozygous β-thal) it will induce a higher Hb F response. This could explain why the same mutations on different β chromosomal backgrounds (some with and others without the *Xmn*1-$^{G}\gamma$ site) are associated with different clinical severity [(66)].

The *BCL11A* gene encodes a zinc finger transcription factor that is a critical modulator of hemoglobin switching and γ-gene silencing, and appears to do so by binding to the locus controlling region as well as the

intergenic region within the β-gene cluster and not to the γ-gene promoter [67, 68]. The myeloblastosis oncogene (*MYB*), on the other hand, is a proto-oncogen that encodes for a c-*MYB* transcription factor playing an essential role in erythroid differentiation and has been shown to modulate Hb F levels in healthy as well as those with hemoglobinopathies [69]. These two QTLs appear to be directly regulated by another key transcription factor, the Kruppell-like factor 1 (KLF 1), and they cooperate with DNA methyltransferase 1 to achieve fetal to adult hemoglobin switch [70, 71].

The role of increased Hb F response as an ameliorating factor becomes evident in patients with homozygous β^0 thal who are mildly or moderately affected despite having minimal level or no Hb A ($\alpha_2\beta_2$), and without α-thalassemia [72].

Other Hb F determinants within the β-cluster gene is related to the nature of mutation itself. Small mutation or deletions that affect the promoter sequence of the β-globin gene are associated with variable increases in Hb F and unusually high Hb A2 levels, which reflects the competition between γ and β-globin gene for interaction with upstream β-LCR. Hence, although such deletions cause a complete lack of β-globin product, the disease severity is ameliorated by the concomitant increase in Hb F [40].

1.2.6.3 Tertiary Modifiers

Variation in the phenotype with regard to some complications have been revealed to be affected by genetic variants. Studies have demonstrated that the degree of jaundice and the incidence of developing gall stones in β-thal is related to polymorphic variant in the promoter of uridine diphosphate

glucouronosyltransferase 1A (UGTIA1) gene, also known as Gilbert's disease [73, 74].

Bone mass is a quantitative trait known to be under strong genetic control involving multiple loci, including estrogen receptor gene, vitamin D receptor (*VDR*) gene [75], collagen type α1 genes [76], and transforming growth factor β1 (*TGFB*1) [77].

A common complication of β-thal involve organ damage due to the iron overload, not just from blood transfusion, but also from increased intestinal iron absorption. A set of genes related to iron metabolism includes; human hemochromatosis (HFE), transferrin receptor 2 (TFR2), ferroportin (FPN), Hepcidin (HAMP) and Hemojuvelin (HJV) genes also influence the different degree of iron loading in β-thal [78].

B-thal patients, especially major and intermedia can develop complications in adult state such as hypercoagulable states. Genetics features involved in thromboembolic events should be considered such as Factor V Leiden (FVL) rs6025, Prothrombin G20210A, and MTHFR mutations [79]. Finally environmental factors, such as malaria infection, may also play an important role in modifying the β-thal phenotype [80].

1.2.7 Clinical Features

β-thal major usually presents in the first year of life, with failure to thrive, poor weight gain and growth, developmental delay, and transfusion dependency for life. The parents may have notice that the infant is pale and jaundiced, with protruding abdomen due to the enlargement of the liver and spleen (hepatosplenomegaly). The later occurs as a result of excessive red cell destruction, extramedullary hematopoiesis (EMH), and later because of

iron overload. The large spleen increases blood requirements by increasing red cell destruction and pooling, and by causing expansion of the plasma volume [81].

On the contrary, β-TI patients present at later age, usually the diagnosis is made at an older age with milder anemia (Hb maintains at or above 7-7.5 g/dL) and by definition do not or occasionally require blood transfusion than TM patients. The clinical spectrum of TI is wide, at the severe end, patients present between the ages of 2-6 years, and although they are capable of surviving without regular blood transfusion, growth and development are retarded. On the other hand of the spectrum, are patients who are completely asymptomatic until adult life, where they develop mild anemia and jaundice, usually associated with a palpable spleen [82].

1.2.8 Associated Complications

1.2.8.1 Hepatobiliary Complications

Among the different organs susceptible to damage in thalassemia patients, the liver represents a major target. Iron overload is the main causative factor, it is associated with the formation of toxic free radicals and damages tumor suppressor genes and DNA repair genes (Figure 1.6). Additionally, iron overload accelerates the process of liver cirrhosis through its profibrogenic effect [83]. Hepatitis viruses, especially hepatitis C virus (HCV) and hepatitis B virus (HBV), are second key factors and appear to work in synergy with iron overload to increase risk of hepatocellular carcinoma (HCC) development, although recently preventive measures have significantly reduced new cases of infection. The potentially aggravating role of hepatotoxic co-factors, such as dysmetabolism and

alcohol, should also be kept in mind. The main risk of chronic liver disease is the development of cirrhosis with its risk of HCC, which is higher in TI as compared to TM, and it is incidence is increasing with age [84, 85].

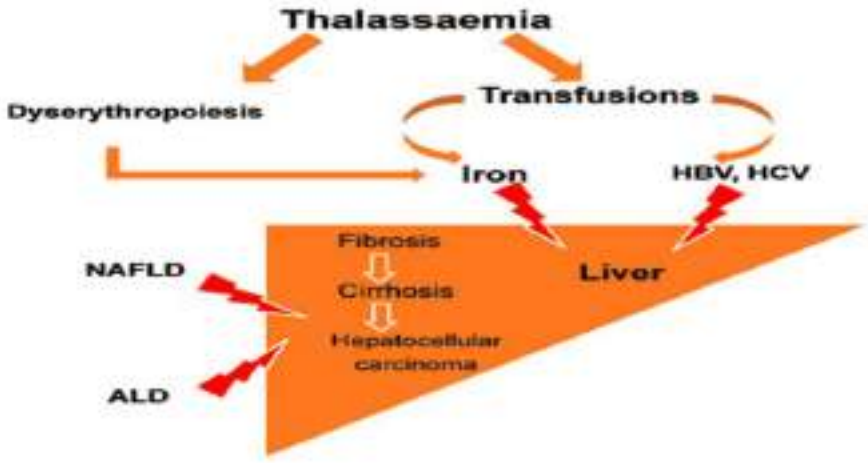

Figure 1.6: Main causes of hepatic iron damage in thalassemia. NAFLD, non-alcoholic fatty liver disease; ALD, alcoholic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus [12].

Due to the above mentioned risks, screening of iron overload was recommended, and through non-invasive quantification of liver iron concentration (LIC) with R2 MRI than the older invasive liver biopsy [86]. However, the widely available and inexpensive method of serum ferritin measurement remains the most heavily used method, particularly in under-resourced area where MRI is unavailable, despite frequent underestimation of the actual iron burden in TI patients. Additionally, TI patients with HCV infection, HBV infection, serum ferritin ≥1000 ng/ml, LIC ≥5 mg Fe/g dry weight (dw), or advanced cirrhosis, are recommended to undergo biannual hepatic ultrasound assessment for HCC screening [87].

Gall stones are a frequent complication of chronic hemolytic anemia, and symptomatic gall stone should be treated with cholecystectomy. In a study,

cholelithiasis was found in 20.3% of patients with TM, and in 57% of those with TI, with the inheritance of Gilbert mutation further increases the risk [88]. Additionally, in the absence of symptoms of cholelithiasis, the gall bladder should be inspected during splenectomy and intervention should be considered as splenectomized patients are at high risk of developing cholecystitis [19].

1.2.8.2 Bone Disease

Bone abnormalities, including facial bone deformities, protrusion of the upper jaw, obliteration of maxillary sinuses, spinal deformities, scoliosis, nerve compression, spontaneous fractures, osteopenia and osteoporosis. Osteoporosis, appears to be more marked in TI as compared to TM, as a result of the enhanced ineffective erythropoiesis and consequent bone marrow expansion [89].

According to the World Health Organization, osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequential increase in fracture risk. It is a prominent cause of morbidity in patients with TM [12]. In TI patients are at high risk of developing osteoporosis with splenectomy, iron overload, low fetal Hb levels, and female gender [90-92]. In contrast, lower rates of osteoporosis were observed in patients on iron chelation therapy and hydroxyurea treatment [90].

The most recent guidelines by the Thalassemia International Federation (TIF) recommend that all patients ≥10 years of age should be screened by yearly assessment of lumbar spine, femoral neck, and distal ulna bone

mineral density (BMD) by using Dual Energy X-ray Absorptiometry (DEXA) [10].

Bisphosphonate is the gold standard of treating thalassemia-associated osteoporosis in both TM and TI. In addition, calcium and vitamin D supplementation is frequently used, although it's efficacy has not been fully established [43, 93].

1.2.8.3 Cardiac Disease

Cardiac disease is the major cause of death in both TM and TI patients [94, 95]. The main finding in TM is iron overload leading to left ventricular (LV) dysfunction, cardiac failure, and cardiogenic death, while most cardiac disease in TI are related to chronic right heart failure secondary to pulmonary hypertension (PHT) [96, 97].

Iron deposition in the heart is more prevalent and happens at a faster rate in TM patients as compared to TI patients [49]. High cardiac output (CO), increased pulmonary vascular resistance (PVR), and PHT are the most significant cardiac findings in TI when compared to TM [94, 96, 97]. Increased CO stems from chronic anemia/hypoxia and related shunt development as a result of increased intramedullary and extramedullary erythropoiesis. In addition, increased oxygen (O2) affinity of the Hb F and blood vessel dilatation secondary to coexistent elastic tissue injury may also act as contributing factors [98, 99].

1.2.8.4 Pulmonary Hypertension

Pulmonary hypertension is a complication of disease progression in the absence of transfusion therapy [96]. A study reported PHT in 66% of TM

patients who had inadequate transfusion therapy, documenting the impact of long-term hypoxia [100]. TI patients are 5 times more likely to have PHT than TM patients. The risk factors are splenectomy, naivety to iron chelation therapy, naivety to hydroxyurea treatment, naivety to blood transfusion therapy, a nucleated red cell count over 300 x 10^6 /L, a history of previous thromboembolic events, and older age [101, 102]. The negative effect of hemolysis on nitric oxide and arginine availability has been implicated [103].

1.2.8.5 Endocrine Disorders

Endocrine abnormalities are among the most common complications of TM. Despite early establishment of appropriate chelation therapy, problems such as delayed growth, sexual maturation and impaired fertility may persist [12], (Figure 1.7).

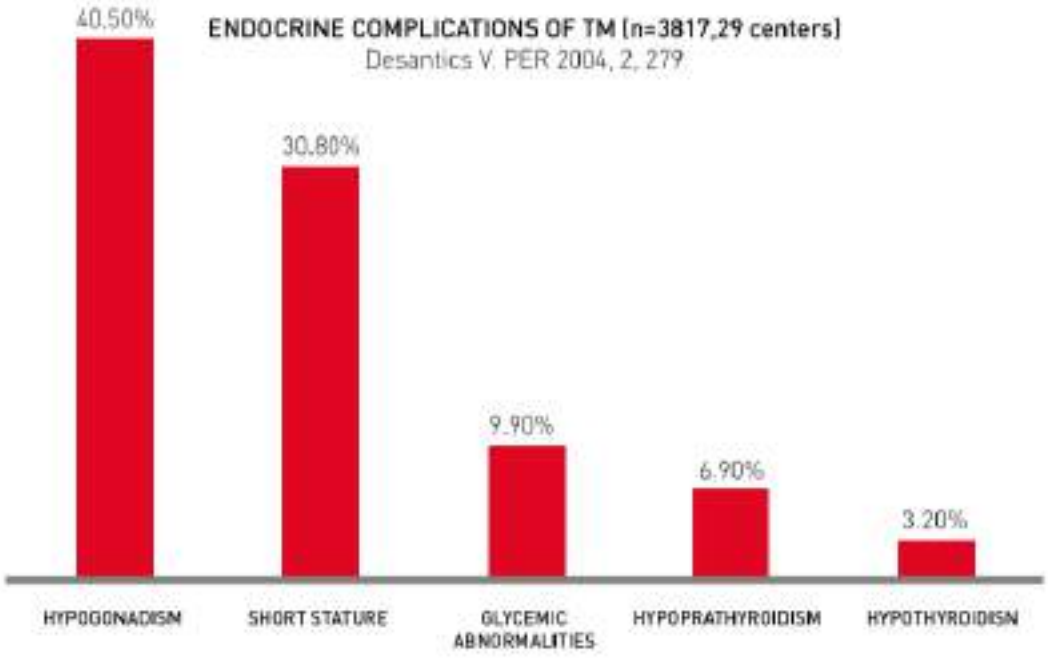

Figure 1.7: Growth and endocrine complications in thalassemia [104].

In TI when compared with TM, the lower prevalence of endocrine disease may be attributed to the lower extent, slower rate and hepatic predominance of iron loading [105].

Iron accumulation results in dysregulation of the hypothalamic-pituitary axis with subsequent multiple endocrine disorders. Hypogonadism is the most frequent, followed by diabetes and hypothyroidism [106]. Furthermore, the prevalence of endocrinopathies in TI increases with age [49].

It is recommended by TIF to run the following tests annually in all TI patients ≥10 years: calcium, phosphate, 25-hydroxyvitamin D, free thyroxine, thyroid stimulating hormone (TSH), fasting plasma glucose, and adrenocorticotropic hormone stimulation test [107].

1.2.8.6 Thrombosis

The existence of chronic hypercoagulable state has been demonstrated in TI. Thromboembolic event is more highly prevalent in TI patients as compared to well transfused TM patients. In a report on 8,860 thalassemia patients from the Mediterranean countries and Iran, thromboembolism occurred 4 times more frequently in TI than TM [108]. Also, an Italian study reported a high prevalence of venous thrombotic events in TI patients [109]. Independent risk factors are splenectomy, serum ferritin level ≥1000 ng/ml, Hb level <9 g/dL, and age >35 years [90, 109, 110].

It has been suggested that the presence of a chronic hypercoagulable state could be due to the procoagulant effect of the anionic phospholipids on the surface of the damaged circulating red blood cells, increased number of platelets, and to abnormal plasma coagulation. Furthermore, the co-inheritance of thrombophilic mutations increased the risk thrombosis [111].

1.2.8.7 Leg Ulcer

Thalassemia intermedia patients have higher risk of developing leg ulcers, particularly in poorly controlled disease, as compared to the regularly transfused TM patients [112]. The typical location is at the medial and lateral malleoli, and they are mostly seen during the second decade of life, with increasing risk with age [80, 113].

The underlying pathophysiology is multifactorial, mainly chronic anemia and hypercoagulability. In addition, elevated venous pressure due to liver injury or right heart failure, with RBC membrane defects and rigidity contribute to poor tissue oxygenation and render the skin injury-prone to minimal trauma [113, 114].

1.2.8.8 Extramedullary Hematopoiesis

The extramedullary hematopoietic masses occur almost exclusively in TI patients compared to TM (particularly when transfusion is inadequate), 20% vs. <1% [90, 115]. Physiologically, regular blood transfusion can decrease the development of extramedullary hematopoiesis (EMH); thus, resulting in relative inactivity of these tissues, and leading to shrinkage of any possible mass [116].

Expansion of hematopoietic tissue in response to ineffective erythropoiesis can involve the reticuloendothelial system resulting in pseudotumors in the liver, spleen, and other sites [117]. Risk factors include, older age, male, and low Hb F levels [49, 118].

1.2.8.9 Renal Disease

Chronic anemia and hypoxia results in tubular cell dysfunction, progressive renal damage and glomerular dysfunction. Iron overload has also been suggested as a prominent player in tubular and glomerular dysfunction. End-stage kidney disease is possible as a consequence of anemia and iron overload-mediated kidney damage [119, 120].

1.2.8.10 Infection

Infection represents the second cause of death in patients with TM. Thal intermedia patients are also exposed to a high risk of infection. Predisposing factors include anemia, iron overload, splenectomy, and a range of immune abnormalities. The reduced formation of nitric oxide in the presence of iron contributes to the risk of infection and iron has also an inhibitory effect on the activity of inferon gamma. As a consequence, iron-loaded macrophages loss the ability to kill intracellular pathogens via the interferon gamma-mediated pathways. Encapsulated organisms frequently found in splenectomized patients are readily controlled by prophylactic vaccination and prophylactic antibiotic treatment. Conversely, infections due to ferrophilic organisms, such as Yersinia and Klebsiella are increasingly being reported in patients chelated with deferoxamine [121].

1.2.9 Laboratory Findings

1.2.9.1 Hematological Findings

The complete blood count (CBC) shows a variable degree of anemia. The red cell indices; mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are low, with a wide red cell distribution width

(RDW). The peripheral blood smear shows hypochromic microcytic red cells with variable degree of poikilocytosis (speculated tear-drop cells), target cells, basophilic stippling, and numerous erythroblasts. The number of erythroblasts is related to the degree of ineffective erythropoiesis, and is markedly increased after splenectomy [122, 123], (Figure 1.8).

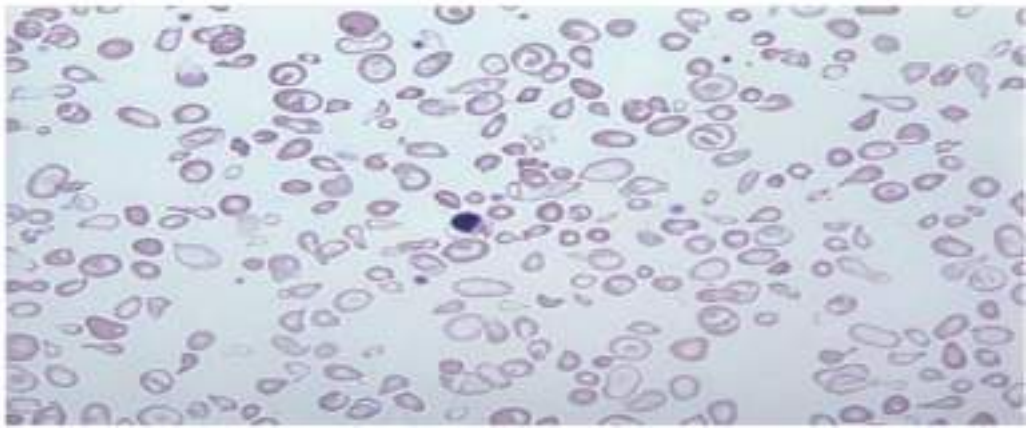

Figure 1.8: The peripheral blood film in β-thalassemia [35].

The reticulocyte count is elevated but less than expected for the degree of anemia, and except in the presence of hypersplenism the white cell count and platelet counts are usually normal. Bone marrow examination is not essential to make the diagnosis, but if performed shows marked erythroid hyperplasia, and many of the red cell precursors contain inclusion bodies with dyserythropoiesis [122, 123].

Hemoglobin Constitution

Hemoglobin analysis is performed by using protein-based techniques such as electrophoretic or chromatographic techniques. In the classical form of β-thalassemia major (homozygotes β°), at hemoglobin analysis, Hb A is absent and Hb F represents the 92–95% of the total hemoglobin. In thalassemia major forms due to double heterozygosity of β°/β^{+}, the Hb A

levels can be variable between 10 and 30% and Hb F between 70 and 90% [123].

The clinical spectrum of thalassemia intermedia is very wide as well as the hematological phenotype including Hb constitution, which are dependent on the underlying extraordinary diverse genotypes. Patients with milder forms may have mild-moderate anemia, and the levels of Hb A and Hb F are very much dependent on the underlying molecular defects and the degree of ineffective erythropoiesis. In TI that results from the homozygous state for severe β^0 ($\beta^0\beta^0$) thal mutations the level of Hb F tends to be the highest and Hb A_2 the lowest as compared with other genotypes ($\beta^+\beta^+$ or $\beta^+\beta^0$). Furthermore, homozygous or compound heterozygous TI state that results from mutations in the promoter region of the β globin gene, unexpectedly presented with high Hb F level, usually in excess of 50% of the total Hb, as well as high Hb A_2 level [123].

1.2.9.2 Molecular Diagnosis of β-Thalassemia

Molecular genetic testing serves an important role in identifying individuals carrying thalassemia trait that can cause adverse outcome in offspring. Furthermore, prenatal genetic testing can identify fetuses with severe globin phenotypes [124]. Hemoglobinopathies which include thalassemia and sickle cell disease, were the first genetic diseases to be characterized at the molecular level and consequently have been used as a prototype for the development of new techniques of mutation detection. There are many different polymerase chain reaction PCR-based techniques that can be used to detect β-globin gene mutations, including dot blot analysis (DB), reverse dot blot (RDB) analysis, the amplification refractory

mutation system (ARMS), restriction endonuclease-PCR (RE-PCR) analysis, Gap-PCR, and direct sequencing. Recently, more sophisticated techniques such as real-time PCR and oligonucleotide microarray analysis have been used for the rapid analysis of thalassemia, however these techniques are more laborious and expensive than the current screening tests (RDB and ARMS). Each method has its advantages and disadvantages, and the particular one chosen by a laboratory for the diagnosis of point mutations depends upon factors such as the technical expertise available in the diagnostic laboratory, the type and variety of the mutations likely to be encountered in the individuals being screened, as well as the budget available [125, 126]. Most mutations are regionally specific and the spectrum of mutations has now been determined for most at risk populations in each of the four regions (Mediterranean countries, Asian-Indian, Southeast Asian, and African). Countries without a large multi-ethnic immigrant population have just a few of the common mutations together with a large and more variable number of rare ones [127]. The diagnostic strategy in many diagnostic laboratories screening for a limited mutation spectrum is to use a simple and cheap PCR technique that allows the detection of the common mutations simultaneously based on allele specific oligonucleotide hybridization or allele specific priming, such as reverse dot-blotting (RDB) or ARMS. This approach will identify the mutation in more than 90% of cases and then a further screening for the known rare mutations will identify the mutation in most of the remaining cases. Mutations remaining unidentified after this second screening are treated as unknown mutations and then characterized by DNA sequencing [128, 129].

Allele Specific Oligonucleotide-Prob Method

The first PCR based method to gain widespread use was the hybridization of allele-specific oligonucleotide probes (ASOs) to amplified DNA bound to nylon membrane by dot-blotting [(130)]. It was first described by Kafatos et al in 1979 [(131)]. Although still in use, the method is limited by the need for separate hybridization steps to test for multiple mutations. This was overcome by the development of the reverse dot-blotting technique, in which amplified DNA is hybridized to a panel of mutation specific probes and normal probes fixed to a nylon strip. This technique is compatible with the optimum strategy for screening β-thal mutations, using a panel of the commonly found mutations for the first screening and a panel of rare ones for the second screening [(132)]. Allele specific hybridization screening is the only technique for the diagnosis of β-thal mutations have been developed commercially and there are currently two competing systems on the market. Vienna Lab have a strip assay using allele-specific oligonucleotide probes which reverse-hybridize to biotinylated DNA. The assays cover 22 β-thal mutations, optimized in separate strips for the common Mediterranean, Middle Eastern and Indian/Southeast Asian mutations. Bio-Rad Laboratories has developed a different system with the oligonucleotides complementary to mutant and normal sequences immobilized on the wells of a microplate. There are two kits for β-thal, one for the eight most common Mediterranean mutations and one for the eight most common Asian β-thal mutations [(133)].

The principle of dot blot (DB) and reverse dot blot (RDB) methods is that a single-strand DNA molecule of defined sequence (the "oligoprobe") can hybridize to a second DNA molecule that contains a complementary sequence (the "target"). In both methods specific amplified fragments of DNA are need to be hybridized with ASO probes that are fixed on the surface of a membrane (RDB) (Figure 1.9) and (Figure 1.10), or by a radioactive-labelled probe to DNA samples fixed to the membrane in the form of dots (DB) (Figure 1.11). DB needs different hybridization and wash temperatures for each probe per mutation, and screens many samples for one mutation per membrane. RDB is a non-radioactive method, which requires the same temperature for hybridization and washing, it can screen one sample for many mutations per membrane. Reverse hybridization technique is fast, cost-effective, and more reliable technique that can reduce false-negative results than the traditional ARMS method [130, 132].

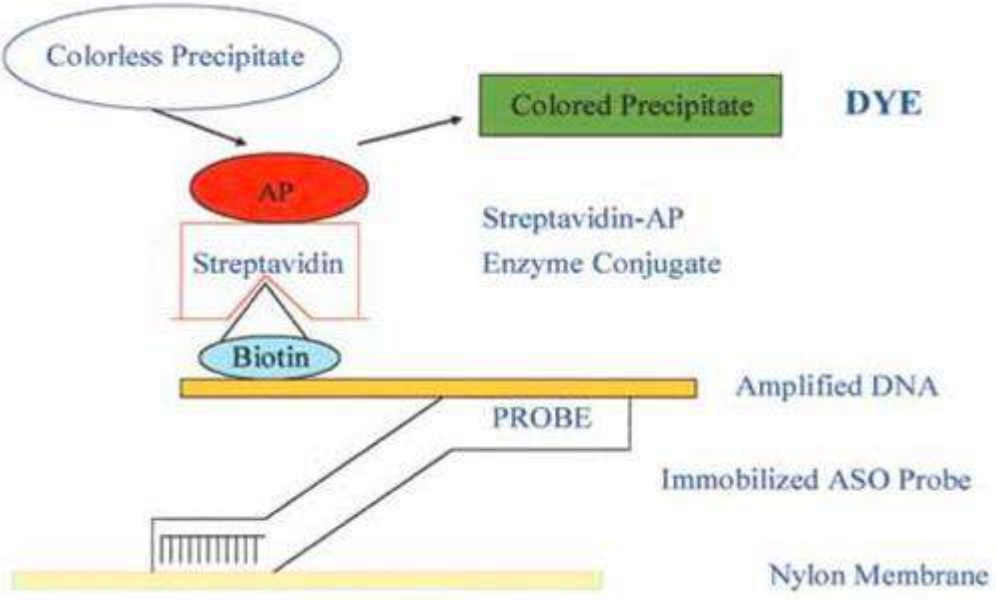

Figure 1.9: The main steps involved in "Reverse Dot Blot Analysis" [133].

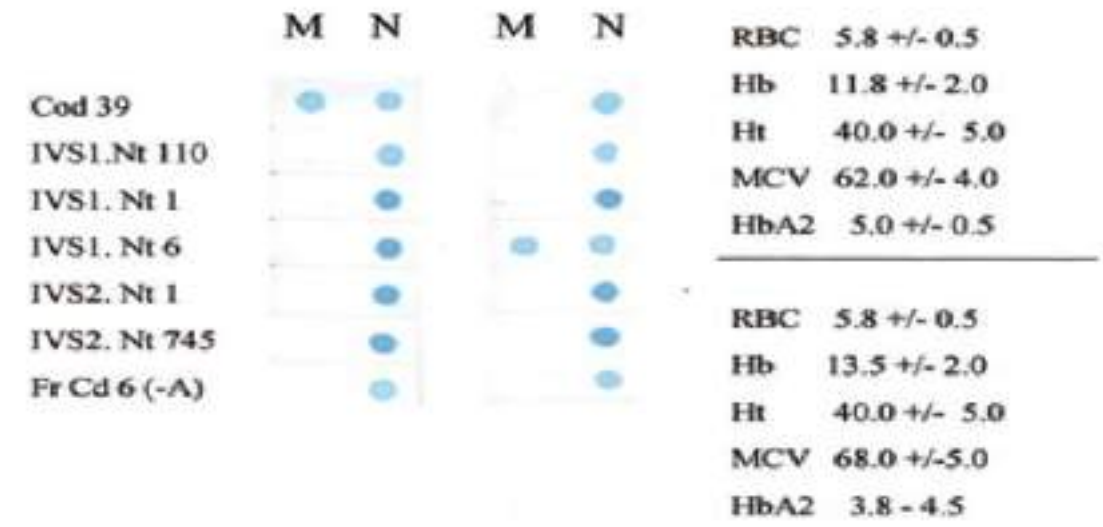

Figure 1.10: An example of a reverse dot-blot analysis that shows two individuals tested for the presence of the most common Mediterranean mutations. On the left is a carrier positive for the Cd 39 (C→T) point mutation, while on the right a IVSI-6 (T→C) point mutation is identified [(133)].
N: normal oligonucleotides;
M: mutant oligonucleotides.

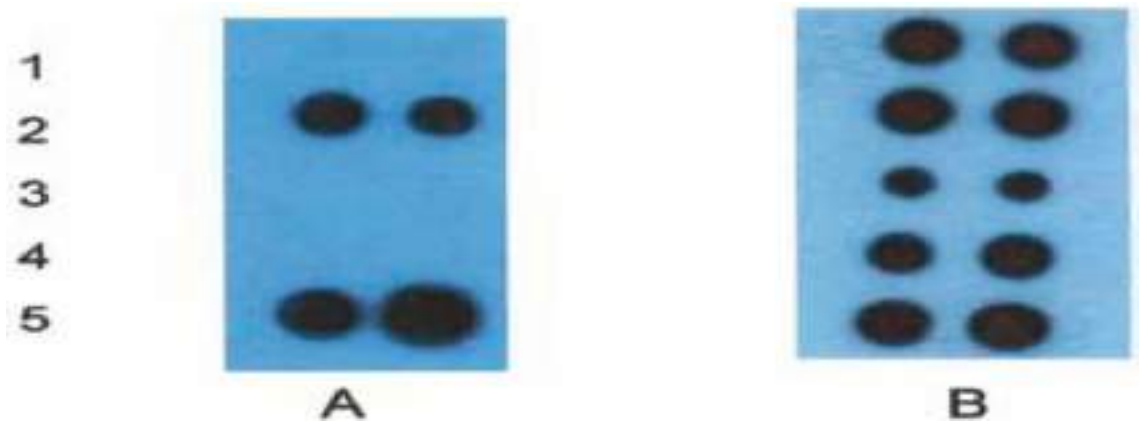

Figure 1.11: An example of dot-blot analysis. Autoradiogram A was performed with IVS1-110 (G→A) mutant probe. Autoradiogram B was performed with ISV1-110 (G→A) normal probe. In autoradiogram A. samples 2 & 5 are positive for ISV1-110 (G→A) mutation [(133)].
Individuals 1, 3, 4 are negative for the IVS1-110 (G→A) mutation.
Samples 2 & 5 are heterozygous for IVS1-110 (G→A).

Amplification Refractory Mutation System (ARMS)

The ARMS technique for detecting known point mutations was first described by Newton et al in 1989 [134]. It has been developed for the diagnosis of all the common β-thal mutations found in all the main ethnic groups [135]. It is a simple PCR-based system that discriminates between normal and mutant alleles by selecting allele-specific primers that have nucleotide at their 3'end corresponding to either normal or mutant sequence. PCR amplifies a DNA fragment only if there is perfect match with the genomic sequence to which the primer is annealing (Figure 1.12). The PCR product then load by electrophoresis on a 3% agarose gel containing ethidium bromide and the visualization by ultraviolet illumination. This method provides a quick screening assay that is cheap and does not require high technology or dedicated instruments. It can be multiplexed for screening multiple mutations in a single PCR assay in one patient but it needs good experience to understand false negatives [136, 137].

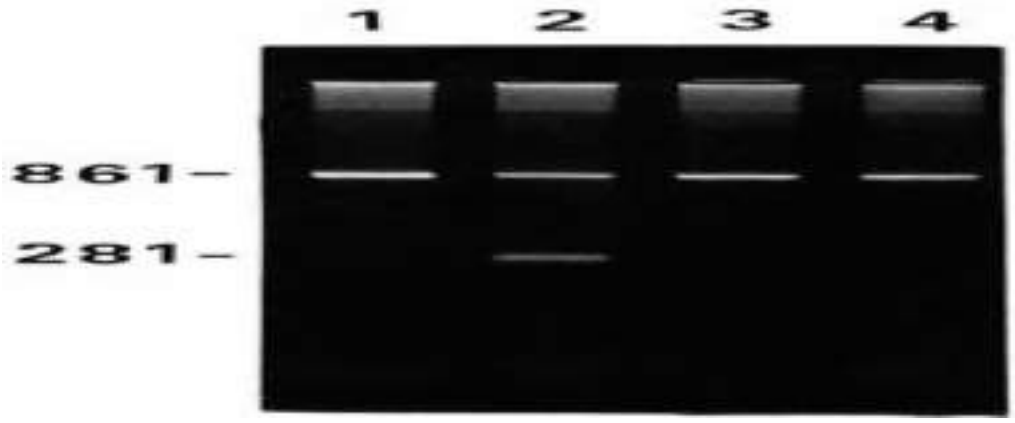

Figure 1.12: An example of screening for common β-thalassemia mutations by ARMS-PCR. Ethidium bromide stained gel showing the screening of a DNA sample for four common Asian Indian β-thalassemia mutations by ARMS-PCR: track1, IVSI-5 (G→C); 2, IVSI-1 (G→T); 3, Cd 41/42 (-TCTT); 4, Cd 8/9 (C→T). The results show the patient carries the mutation IVSI-1 (G→T) [133].

The diagnostic characteristics of the reverse hybridization and ARMS technique are shown in Table 1.2.

Table 1.2: Comparison of different factors determining the efficiency of ARMS and reverse hybridization in beta thalassemia diagnosis [(9)].

	ARMS	Reverse hybridization
Turnover time	several days	6-8 hours
Specificity	High	High
Reaction reproducibility	Depending on many factors	Very high
Number of PCR reactions per sample	8-88	1
Number of mutations detected per test	1	25 or more (depending on the test strip)
Documentation	Requires documentation process after experiment	Self-documented
Technician time (number of patients: time in days)	1:1	10:1
Starting material	Depending on the number of PCR reactions	0.5 µg genomic DNA for just one PCR reaction
Toxic materials	Ethidium bromide (carcinogen)	None
Equipment	Expensive (large PCR machine, gel electrophoresis, photodocumentation system)	Less expensive (small PCR machine, agarose gel, small shaking water bath)

Restriction Enzyme Analysis

It is a rapid, simple and reliable method used if a mutation happens to create or abolish a restriction site. The limitations of this method that the majority of globin gene mutations do not create or destroy a restriction endonuclease recognition site in the globin gene sequence, and some enzymes for those that do are expensive [(138)]. However, the main use of this PCR technique is for the diagnosis of the clinically important Hb Variants Hb S, Hb D-Punjab and Hb O-Arab.

G**ap-PCR**

This is a rapid, simple and non-radioactive method that allows the identification of DNA deletions or gene rearrangements. The limitation of this method is that the deletion endpoints must be known to design the primers [139].

The ASO dot-blot analysis, ARMS analysis, and RE-PCR are known to be difficult, time consuming and more liable to contamination [9, 129]. The RDB hybridization StripAssay method is characterized by rapid turnover time, very high reaction reproducibility, one PCR reaction, less contamination and can detect 22 or more mutations per test (depending on the test strip). Only very small quantities of DNA are required and the test is not expensive [9, 140].

The advantages and disadvantages of each method of analysis of known mutations are summarized in Table 1.3.

Table 1.3: Advantages and disadvantages of different molecular methods for detection of known β-globin mutations.

Advantages	Disadvantages
ASO dot blot hybridization • Widely applicable and reliable	ASO dot blot hybridization • Traditional protocols use radioactively labelled probes • Time consuming and can only screen one mutation at a time • Expensive
Reverse dot blot hybridization (RDB) • Simultaneous screening for many mutations • Usually no radioactivity • Relatively inexpensive • Simple, rapid and reliable	Reverse dot blot hybridization (RDB) • Need sample controls to standardize new mutations • Need good technical expertise in the laboratory to set up and validate RDB • May be expensive if use commercial kits
ARMS-PCR • Simple, rapid and inexpensive • Suitable for technical modification • Can be multiplexed to detect mutation	ARMS-PCR • Need control DNA to validate test and some rare mutations unavailable in homozygous state • Primers can degrade, giving non-specific signal
Restriction enzyme (RE)-PCR • Simple and rapid • Reliable	Restriction enzyme (RE)-PCR • Limited to few mutations • Need care to avoid partial digestion problems • Some enzymes costly
Gap-PCR • Simple, rapid and inexpensive • Can be multiplexed to detect mutation	Gap-PCR • Need control DNA to validate test • Limited to diagnosis of deletions with known DNA breakpoint sequences

1.2.9.3 Assessing Iron Status

Several methods are currently available for the diagnosis and monitoring of iron overload in patients with TM and TI. Serum ferritin assessment is widely available and might be the only assessment that is affordable in resource-poor countries. The assessment of serial serum ferritin concentration can be a good indicator of iron chelation effectiveness, and available guidelines recommend measurement of serum ferritin concentration every 3 months [10, 12, 141].

In patients with TM, maintaining concentrations lower than 1000 μg/L of ferritin is associated with lower morbidity and mortality, and this threshold is most commonly used to indicate the need for initiation of iron chelation therapy [12, 15, 142]. Serum ferritin concentrations consistently higher than 2500 μg/L are associated with an increased risk of cardiac and endocrine disease, and thus are commonly used to flag the need for dose optimization or a change in iron chelation therapy [12, 142]. Moreover, single measurements of serum ferritin concentration are prone to mis-interpretation or not being representative of a patient's condition, considering their high variability when a patient is experiencing inflammation, infection, vitamin C deficiency, hepatic dysfunction, and severe iron overload with values higher than 4000 μg/L [12, 143].

In patients with β-TI, concentrations of greater than 800 μg/L are associated with an increased risk of morbidity, and concentrations of less than 300 μg/L are associated with an absence of risk of morbidity. These thresholds are thus used to indicate the need for iron chelation initiation or interruption [10, 144].

Measurement of liver iron concentration has become common practice since the early 2000s, considering the strong correlation with total body iron stores [145]. Initial reports relied primarily on the use of liver biopsy, although the technique is now used less frequently and only reserved for those situations when assessment of hepatic histology is needed [146]. A significant correlation between serum ferritin and LIC has been established in regularly transfused patients with TM [147, 148]. However, in patients with TI the correlation between serum ferritin and LIC has proved equivocal, with some studies demonstrating a statistically significant correlation [87, 149], and others showing no notable correlation [150]. The use of a Superconducting Quantum Interference Device (SQUID) is also still practiced, but the technology is only available in few centers worldwide [12, 151].

The introduction of MRI for the non-invasive assessment of liver iron concentration, and later myocardial iron concentration, is considered one of the most important advances in thalassemia care over the past decade. The use of MRI in clinical practice can allow better tailoring of iron chelation therapy. For measurement of liver iron concentration, both the R2 and T2* techniques are used as they have been validated against liver biopsy measurements of iron [152]. They are internationally reproducible and accurately measure liver iron concentration, and give a measurement of liver iron concentration in mg of iron per g of dry liver weight [153]. They are also equally effective in evaluating chronic response to iron chelation [154].

1.2.10 General Management

Without intervention, any form of thalassemia (except carrier state) is a progressive disease with increased morbidity as the patient advances in age [49]. Moreover, the availability of effective therapeutic options and improved patient survival could allow multiple morbidities to manifest with age and the quality of life of the patient to deteriorate [155].

Management guidelines for both patients with TM and TI are available as part of the global efforts of the Thalassemia International Federation (TIF) to improve the outcomes of patients affected by these disorders [10, 12].

There are a number of treatment modalities currently available for managing patients with thalassemia, including transfusion therapy, iron chelation therapy, splenectomy, modulation of Hb F production, and hematopoietic cell transplantation.

1.2.10.1 Blood Transfusion

Transfusion therapy works by supplying normal erythrocytes for correcting anemia and suppressing ineffective erythropoiesis, essentially controlling all downstream pathophysiological mechanisms in thalassemia [156]. Advances in transfusion and iron-chelation therapy were associated with improvement in survival in long-term follow up. Blood product screening, preparation and administration practices have largely improved over the past 3 decades [157], and nowadays most patients with TM can successfully achieve target Hb concentration of 9.0-10.5 g/dL (11.0-12.0 g/dL in those with heart disease); although access to blood for transfusion therapy remains a challenge in resource-poor countries and poses an important public health burden [12].

Transfusion are sporadically given to TI patients under acute stress or experiencing a drop in Hb concentration, such as growth failure, poor performance at school, pregnancy, infection, symptomatic anemia, surgery, and evidence of complications, such as heart failure, PHT or leg ulcer [(115)]. Some patients are maintained on a regular transfusion programme following these incidents, a practice that in many cases might not be clinically indicated. Hemoglobin concentration in isolation should not be an indication for lifelong, regular transfusions in patients with TI, particularly many patients can adapt to their chronic anemia without substantial bone marrow stress [(158)].

In a study, TI patients who were placed on transfusion regimens (intermittent or regular) suffered fewer complications relevant to chronic anemia, ineffective erythropoiesis and hemolysis (mainly EMH, PHT and thrombosis); while suffering from a higher rate of iron overload related endocrinopathy [(90)]. Transfusion therapy, however, has it's own side effects, although the risks of alloimmunization and blood born infection remain a concern in transfused patients, the greatest challenge with regular transfusion therapy is secondary-iron overload [(10, 12)]. Alloimmunization is a relatively common observation in TI, and the risk is decreased if transfusion therapy is initiated before the age of 12 months. Thus, early introduction of transfusion therapy will also help alleviate the increased risk of alloimmunization [(159)].

It is strongly suggested that transfusion be started with packed red cells, leucodepleted and matched blood for ABO, D, and Kell antigens. Once treatment decision is taken, transfusions should be applied regularly,

targeting the suppression of the ineffective erythropoiesis and the reduction of native RBCs and hemolysis [160].

1.2.10.2 Iron Chelation Therapy

In patients with TM, maintaining concentrations lower than 1000 μg/L of ferritin is associated with lower morbidity and mortality, and this threshold is most commonly used to indicate the need for initiation of iron chelation therapy [12, 15, 142]. Furthermore, liver iron concentrations greater than 7 mg/g are usually used to indicate increased risks of complication, and lower target concentrations are used to prevent iron related complications [161, 162].

In patients with TI, liver iron concentration values higher than 5 mg/g are associated with increased morbidity, supporting the recommendation of iron chelation initiation in patients showing liver iron concentrations greater than 5 mg/g [10].

Three iron chelators are currently available for the treatment of iron overload in patients with thalassemia: deferoxamine in subcutaneous or intravenous injection, oral deferiprone in tablet or solution form, and oral deferasirox in dispersible tablet and, more recently, filmcoated tablet forms (Table 1.4), and guidelines for their use are now widely available [10, 12, 141].

Table 1.4: Characteristics on iron chelators for the management of iron overload in thalassemia [81].

	Deferoxamine	Deferiprone	Deferasirox
Administration[a]			
Method	Subcutaneous or intravenous	Oral	Oral
Frequency	8-12 h, 5-7 days per week	3 times daily	Once daily
Half-life of iron-free drug[b]	20-30 min	3-4 h	12-16 h
Lipid solubility[a]	Low	Intermediate	High
Route of iron excretion[a]	Urinary and faecal	Urinary	Faecal
Recommended dose[a,c]	20-60 mg/kg per day	75-100 mg/kg per day	TDT: 20-40 mg/kg per day; NTDT: 5-20 mg/kg per day
TIF guidelines indication			
TDT[c]	>2 years: first-line	2-6 years: no sufficient data; >6 years: second-line†	2-6 years: first-line (USA), second-line (EU); >6 years: first-line
NTDT[a]	No sufficient data	No sufficient data	>10 years: first-line
Most relevant clinical data			
TDT[a,c]	Reduction in serum ferritin and liver iron concentration;[a,d] improvement in cardiac T2*;[a,d] improvement in cardiac dysfunction with continuous infusion[a,d]	Improvement of cardiac T2* in monotherapy or in combination with deferoxamine (higher doses than commonly used in clinical practice);[a,c,e,f] improvement in cardiac dysfunction in combination with deferoxamine;[a,f,g] improvement in endocrine dysfunction in combination with deferoxamine or deferasirox[a,c,f,h]	Reduction in serum ferritin and liver iron concentration after up to 5 years, and cardiac T2* after up to 3 years of therapy; even in patients with severe iron overload,[a,c,i,j] not inferior to deferoxamine for improving cardiac T2*;[a,d] improvements in hepatic fibrosis and inflammation;[a,k] stabilisation of heart function;[a,d,l] stabilisation of endocrine function[a,h]
NTDT[a]	Data restricted to case series and small studies	Data restricted to case series and small studies	Significant reduction in serum ferritin and liver iron concentration after up to 2 years of therapy[a,m]
Main adverse events[a,n]	Ocular and auditory symptoms, bone-growth retardation, local reactions, allergy	Gastrointestinal symptoms, arthralgia, agranulocytosis or neutropenia	Gastrointestinal symptoms, increased creatinine, increased hepatic enzymes
Pregnancy[a,o]	Contraindicated (but has been used in third trimester)	Contraindicated	Contraindicated

Data from several large, randomized trials since the development of MRI technology showed the efficacy and safety of oral iron chelation therapy in removing iron from the liver and heart, which represents a considerable advance in patient management owing to the greater convenience compared to parenteral deferoxamine. However, parenteral deferoxamine remains the treatment of choice in patients with decompensated heart disease, and might be the only affordable option in resource poor countries [12].

Adherence to iron chelation therapy correlates with both effective management and patient survival, as is evident from several studies. Moreover, appropriate dose determination and adjustment of the iron chelator according to baseline and ongoing iron intake is essential to ensure adequate response and avoid over chelation [12].

1.2.10.3 Splenectomy

Splenectomy have traditionally been performed as an alternate or adjunct to transfusion therapy. Although some studies have found that splenectomy had resulted in improvements in growth, quality of life, and hemoglobin concentration for some patients [163], the procedure has now become almost obsolete in patients with TM, especially in the context of improved access to and safety of blood transfusions with regards to blood-borne infections. The procedure is still more commonly used in patients with TI in a very specific indications, such as poor growth and development, hypersplenism with symptomatic leucopenia and/or thrombocytopenia, or symptomatic massive splenomegaly [10]. When indicated, appropriate treatment with vaccination and antibiotics is recommended, and prophylaxis with aspirin or low molecular weight heparins for high-risk patients [10, 12].

However, data for serious adverse events caused by splenectomy continues to accumulate in the thalassemia patient population, alongside the known risk of infection and sepsis. Removal of the spleen promotes a hypercoagulable state in thalassemia because of the decreased ability to scavenge procoagulant red blood cells and activated platelets. In splenectomized TI patients, thrombin generation is significantly higher than in control subject and patients who had not undergone splenectomy [109]. Observational studies have further established that splenectomized TI patients are at a 4–5 fold increased risk of overt venous thrombosis and other vascular manifestations like pulmonary hypertension, leg ulcers, and silent strokes than non-splenectomized patients [90, 164].

In patients with β-TI, the long-term risk of thrombosis is further increased in those with concomitant high counts of nucleated red blood cells (≥300 × 10^6 cells per L) and platelets (≥500 × 10^9 platelets per L), and those patients who never received any transfusions [110]. The spleen also usually acts as a reservoir for scavengers of toxic iron in the body, and so removal of the spleen could also decrease the ability of these scavengers on iron free fractions including non-transferrin bound iron (NTBI), which explain the higher serum level on NTBI in splenectomized TI patients, thus putting patients at increased risk of end organ damage [90].

1.2.10.4 Modulation of Fetal Hb Production

Increasing the synthesis of Hb F is potentially applicable to thalassemia intermedia, in that relatively small increase in Hb levels with a corresponding reduction in ineffective erythropoiesis could help a patient thrive who would otherwise require regular transfusions [165]. Several cytotoxic agents with this effect have been identified, including hydroxycarbamide (hydroxyurea) and cytosine arabinoside. Hydroxyurea (HU) is the most widely used drug in this context. Data from observational studies suggest that HU therapy is associated with lower rates of extramedullary hemopoiesis, osteoporosis, leg ulcer, hypothyroidism, and PHT in TI patients especially when combined with transfusion and iron chelation therapy [90]. Direct prospective evidence is still lacking and the clinical benefit of HU therapy has not been systematically established [10]. An increase in Hb level by at least 1 g/dL at 6 months of therapy is considered to be an adequate response, and patient should be evaluated periodically afterward to ensure benefit is maintained and to detect adverse

effect including rashes, alopecia, gastrointestinal disturbances, and myelotoxicity [91].

Erythropoietin (EPO) has also been shown to increase Hb F levels in some patients with TI [165]. Preliminary trials with intravenous and oral butyric acid derivatives have shown increases in fetal and total Hb levels in patients with TI [166].

Thalidomide, a drug known for it is immunomodulating and anti-angiogenic properties, has been demonstrated to induce γ globin gene expression and to increase the proliferation of erythroid cells [167]. Two reports documented the successful treatment of TM patients with thalidomide, where patients achieved an increase in Hb F and total Hb production [168, 169]. In addition, a recent study observed a significant response to thalidomide in symptomatic β-thalassemia [170].

In summary, most trials on agents that modulate Hb F production in TI patients are small, and large, randomized, controlled trials are needed before these agents or their derivatives are widely used in TI management [43].

1.2.10.5 Hematopoietic Stem Cell Transplantation (HSCT)

Replacement of mutant haemopoietic cells using haemopoietic stem-cell transplantation is the only existing curative therapy for thalassemia and is now an established approach to correct defective erythropoiesis, particularly when matched sibling donors are available. Haemopoietic stem-cell transplantation is now widely applied with a disease-free survival exceeding 80% with transplants from human leucocyte antigen (HLA) matched-sibling donors [171]. Moreover, improvements in the management

of graft versus-host disease (GVHD) and inducing graft tolerance have encouraged the use of unrelated donors and umbilical cord blood as the haemopoietic stem-cell source for patients who do not have a matched-sibling donor. Nevertheless, matched-unrelated transplants for high risk patients with thalassemia have an overall survival of 65%. However, a 5–10% mortality from transplant conditioning, GVHD, and graft failure continues to limit the acceptability of this treatment method. The need for complete myeloablation, which can result in infertility and other toxic-effects, is also a concern, although improved approaches with reduced intensity conditioning are being developed [(172)].

1.2.11 Future Interventions

Over the past decade, several therapeutic options have emerged for patients with β-thalassemia. These advances aimed at improving iron dysregulation, globin-chain imbalance, and/or ineffective erythropoiesis.

1.2.11.1 Improving Iron Dysregulation

Minihepcidin, long-acting hepcidin analogs, are currently being studied in clinical trials with hypothesized outcomes including decreasing iron absorption levels from the gastrointestinal tract, regulating iron handling by macrophages, increasing hemoglobin concentration, and reducing spleen size [(173)].

An alternative approach is to stimulate endogenous hepcidin production through the downregulation of a metallo protease, TMPRSS6, that plays a key role in hepcidin expression [(52)]. Antisense oligonucleotides and siRNAs targeting TMPRSS6 have been effectively used to stimulate hepcidin,

reduce the iron burden, and improve in effective erythropoiesis and red blood cell survival [174].

1.2.11.2 Correcting Globin-chain Imbalance

Gene therapy is a promising treatment modality in the management of thalassemia. Some recent studies have described the long-term correction of murine models of human β-thalassemia and sickle cell anemia by lentivirus-mediated gene transfer [175]. The emergence of gene editing technology, whether by direct correction of genetic mutations in the endogenous DNA of the cell or by disruption of specific DNA sequences in the genome, offers a new approach for treating β-thalassemia. This is facilitated by site specific double strand breaks which can be induced with zinc finger nucleases, transcription activator-like effector nucleases, meganucleases, and, more recently, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system [176]. Other alternatives that could allow more efficient and immediate treatment of β-thalassemia with genome editing include the disruption of factors that silence the γ-globin genes, such as *BCL11A* or γ-globin repressive elements within the β-globin gene locus [177].

1.2.11.3 Improving Ineffective Erythropoiesis

Ineffective erythropoiesis is the major contributor to the anemia observed in patients with β-thalassemia. In the past few years, a group of ligand traps (which include sotatercept and luspatercept) have been developed that have shown potent stimulation of late-stage erythropoiesis and improved ineffective erythropoiesis in patients with β-thalassemia [178, 179]. Ongoing clinical studies of these drugs are evaluating their role in reducing

transfusion requirements and raising hemoglobin concentrations in patients with TM and TI, respectively. JAK2 inhibition, an established therapy in patients with myeloproliferative disorders, can also ameliorate ineffective erythropoiesis and decrease spleen size in mice with thalassemia, which has motivated ongoing clinical trials in this area to evaluate the role of JAK2 in patients with TM [(180)].

1.2.12 Prevention

β-thalassemia is an important health problem in Iraq, with an estimated 15,000 registered patients with thal major and intermedia, and 3.7-4.5% of estimated carrier rate in various parts of the country [(2)]. The problem is further aggravated by around 30% consanguineous marriage rate [(181)]. Additionally, access to blood for transfusion therapy in limited-resource countries is a challenge and poses a considerable health burden [(81)]. Such a situation would make initiating a preventive program a necessity.

The determination of the mutations responsible for β-thal in particular populations is an important prerequisite in establishing such a program in that population, because generally a handful of mutations constitute the bulk of these mutations, although the distribution of these mutations varies in various parts of the world and in different ethnic groups [(28)].

A preventive program for hemoglobinopathies based on the concept of pre-marital screening, counselling, and pre-natal diagnosis [(182)], and it was the only viable option in Sulaymaniyah/Iraq to minimize the birth of affected fetuses. Sulaymaniyah Hemoglobinopathies Preventive Program started in 2008 at Sulaymaniyah Public Health Laboratory, and over the first 5 years, the program has succeeded to reduce the birth of affected fetuses by

hemoglobinopathies by 65%, and can possibly serve as a prototype for other regional programs [183]. Furthermore, it was made legally mandatory to perform premarital screening for hemoglobinopathies, and various religious scholars agreed on the principle of allowing selective termination of the affected fetus prior to 16 weeks of gestation [184].

CHAPTER TWO

PATIENTS and METHODS

2.1 Study Design and Ethical Approval

2.1.1 Study Design

This is a cross-sectional study on 242 β-thalassemia patients conducted at Sulaymaniyah Thalassemia Center between June 2018-September 2019. The research work was carried out at the molecular department and patients consultation clinic at Thalassemia Center.

2.1.2 Ethical Approval

All the participants were informed about the study project and consents were obtained, and the present study was commenced after obtaining approval from the ethical committee at the College of Medicine, Sulaymaniyah University, Kurdistan Region of Iraq (approval no.61 on April 16, 2018). All methods were performed in accordance with Helsinki Declaration.

2.2 Patient Enrollment

A total of two hundred and forty-two β-thalassemia patients, including 159 TI and 83 TM patients from 162 families who were registered and received treatment at the Sulaymaniyah Thalassemia Care Center were recruited. The inclusion criteria for both TI and TM were based on criteria previously described [82, 115, 185-187].

The diagnostic criteria for TI were;

1- Age at initial presentation ≥2 years.
2- Maintain Hb level usually between 7-10 g/dL without the need for regular blood transfusions.
3- Variable degree of spleen enlargement.

The diagnostic criteria for TM were;

1- Age at initial presentation < 2 years.
2- Hemoglobin level usually <7 g/dl with regular transfusion requirement.
3- Marked splenomegaly or history of splenectomy.

2.3 Demographic and Clinical Evaluation

All patients have gone through a standardized interview process and their relevant demographics and full medical information including age, gender, parents' consanguinity, family history of thalassemia, age at diagnosis, age of first transfusion (including frequency and time interval of transfusion within the last year), history and age at splenectomy and cholecystectomy. Details regarding the utilization of hydroxyurea treatment and iron chelation therapy (including type of chelating drug) within the last year

were also recorded through a comprehensive questionnaire specially designed for the study.

Physical examination performed to evaluate thalassemic facies, height to judge growth retardation (short stature), defined when height > 2 SD below 3rd percentile for the patient age and gender and the height was determined by growth diagram provided by National Center for Health Statistics (NCHS) [188, 189]. Size of the liver and spleen (non-splenectomized) were also measured, detection of leg ulceration, with screening for gall bladder calculus by ultrasound examination.

Additionally, documented thrombotic events, radiological evidence of extramedullary hemopoiesis and pathological bone fracture, were all retrieved from patient's electronic medical records.

2.4 Bone Mineral Density Assessment

Patients aged ≥10 years were screened for osteopenia and osteoporosis annually by measuring bone mineral density (BMD) using Dual Energy X-Ray Absorptiometry (DEXA, Lunar iDXA DXA System, analysis version: 13.20 by GE Healthcare) scan as recommended by Thalassemia International Federation (TIF) [10]. According to World Health Organization (WHO) criteria, patients with low BMD and a Z-score of ≤ -2.5 SD below the mean for the age and sex-matched control (in patients up to the age of 20 years) and T-score ≤ -2.5 SD (in those aged 20 years or more) were considered as osteoporosis based on determining the BMD (g/cm^2) at the AP lumbar spine (L1-L4) and femoral neck [190-192].

2.5 Echocardiography Assessment

Screening for PHT was performed by periodic echocardiography evaluation for all patients at their regular visiting dates. Patients detected with a mean pulmonary artery systolic pressure (PASP) ≥25 mmHg combined with exertional dyspnea at rest and no evidence of left-side heart failure were diagnosed as pulmonary hypertension [(193)].

2.6 Materials

Molecular work reagents performed using β-globin StripAssay MED® commercial Kit, (Vienna Labordiagnostica GmbH, Vienna), Diagnostic kits consist of the following:

1. Lysis solution (50 ml).
2. Amplification mix (500 µl).
3. Taq DNA polymerase.
4. Taq dilution buffer (500 µl).
5. DNAT (1.5ml).
6. Typing trays (3).
7. Teststrips (20).
8. Hybridization buffer (25 ml).
9. Wash solution A (80 ml).
10. Conjugate solution (25 ml).
11. Wash solution B (80 ml).
12. Color developer (25 ml).

2.7 Instruments and Tools

The instruments used in the present study all belonged to the Molecular Department of Sulaymaniyah Thalassemia Center, and are listed in Table 2.1.

Table 2.1: The instruments and disposables.

No.	Instruments and disposables
1	Biological safety cabinet for DNA extraction
2	PCR biosafety hood/ PCR workstation
3	T100^{TM} Thermal cycler (BIORAD/California, USA)
4	Nano drop spectrophotometer/ for measuring DNA concentration
5	Water bath with shaking platform and adjustable temperature (45°C ± 0.5°C)
6	KBC micro spin and vortex
7	Rotary mixer
8	Microcentrifuge (3,000-12,000 rpm) (Beckman/UK)
9	Shaker (rocker or orbital shaker)
10	Vacuum aspiration apparatus
11	Incubator with heating block, capable of 56°C and 98°C (± 2°C)
12	Stopwatch
13	Ice rack and plain rack for microtubes
14	Eppendorf micropipette (0.5-10µl, 10-100µl, 100-1000µl)
15	Molecular biology grade (nuclease-free) PCR tubes (200µl) and microtubes (1.5 ml)
16	Molecular biology grade (nuclease-free) disposable tips (1000µl, 100µl, 10µl)
17	K_3-EDTA vacuum tubes (3ml)
18	Plain tubes
19	Multiple sample needle and disposable needle holder
20	Disposable gloves
21	Deep freezer (-20°C) and refrigerator (4°C)

2.8 Laboratory Tests Performed

2.8.1 Hematological Investigations

seven ml of blood was collected from each patient using multiple-sample needles and disposable needle holders; 2.5 ml was transferred to K_3- EDTA containing tube and the remaining 4.5 ml to a gel tube. The K_3-EDTA blood samples were used for routine measurement of complete blood count with a fully automated hematology analyzer (Swelab, Spånga, Sweden), and for the estimation of Hb A_2 and Hb F by high-performance liquid chromatography (HPLC) [D-10, Bio-Rad Laboratories, Hercules, CA, USA]. In patients who received blood at a rate of more than 4 occasions/year, the HPLC result at the time of diagnosis was quoted, if available. Then, the remaining blood in EDTA tubes were used for DNA extraction immediately or frozen at -20 for later DNA processing. These investigations were performed just before to the next blood transfusion.

2.8.2 Biochemical and Other Investigations

Serum was used to estimate ferritin level by Enzyme- Linked Immunosorbent Assay (ELISA) (Biokit-USA), and liver function test (LFT) (alanine transaminase; ALT). Blood glucose (mg/dL) for detection of diabetes mellitus (DM), in which the diagnosis was established in patients who were under insulin therapy, also in individual with fasting blood sugar level ≥126 mg/dl on 2 separate occasions according to American Diabetes Association, WHO criteria and National Diabetes Health Group [194, 195].

Virological tests; Hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV) antibodies and Human Immune Deficiency Virus (HIV)

antibody 1 and 2 (Plasmatic Laboratory Product-UK). Thyroid function test (Thyroid-stimulating hormone TSH and free T4), using enzyme immunoassay (TOSOH-Japan) was estimated in patients ≥ 10 years annually [(10)]. Subclinical hypothyroidism was diagnosed based on high TSH >4.7 μIU/ml combined with normal free T4 >0.8 ng/dl, while patients with high TSH >4.7 μIU/ml and low free T4 <0.8 ng/dl were diagnosed as overt hypothyroidism [(196)].

2.8.3 Molecular Investigations

Molecular identification of β-globin gene mutations (Mediterranean type) using polymerase chain reaction (PCR) and reverse-hybridization with β-globin StripAssay MED® Kit (Figure 2.1), (Vienna Labordiagnostica GmbH, Vienna, Austria) was performed.

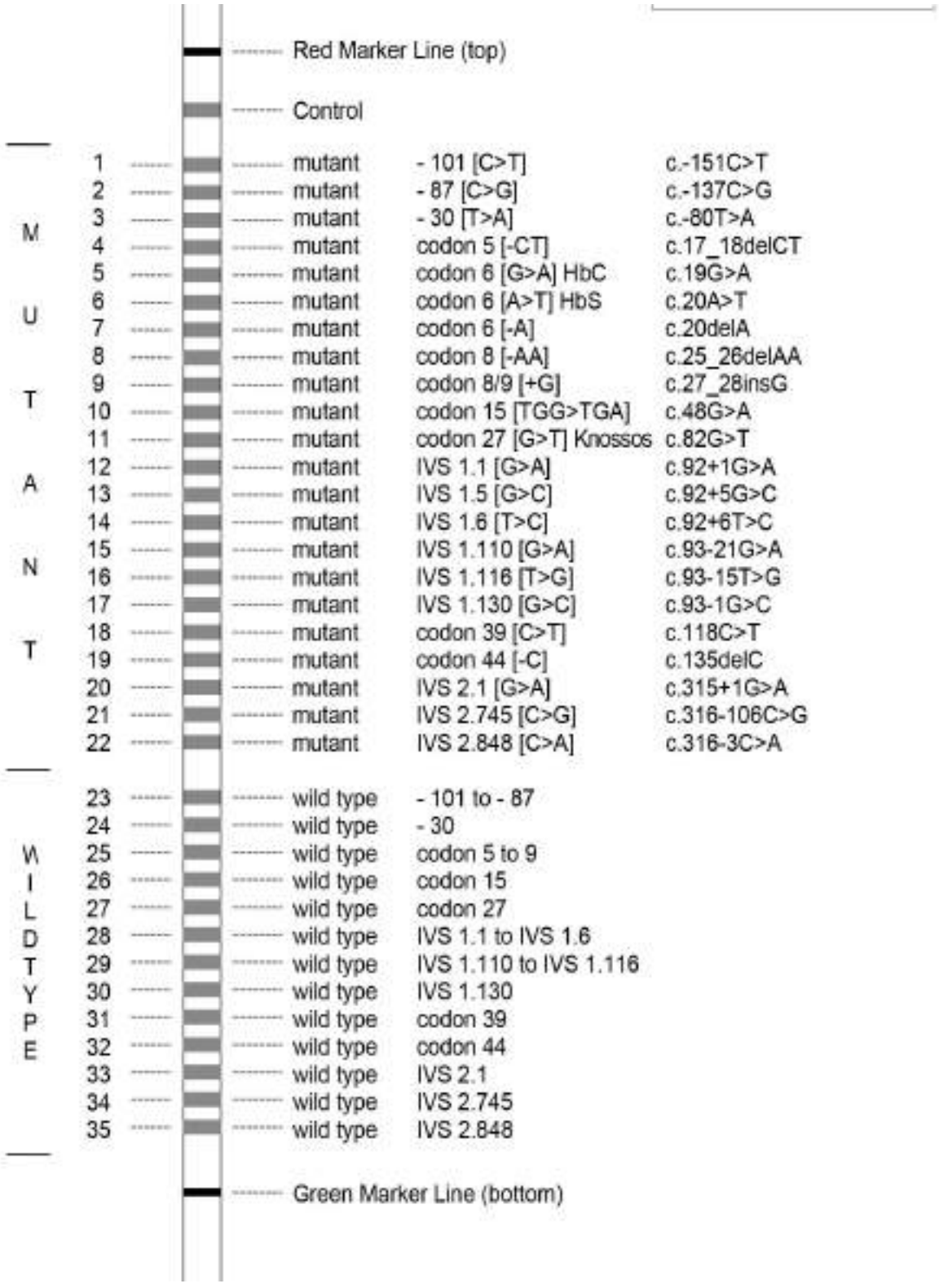

Figure 2.1: Mediterranean TestStrip design of ViennaLab.

The assay covers 22 Mediterranean β-globin mutations:

101 (C>T), -87 (C>G); -30 (T>A); codon 5 (-CT); codon 6 (G>A); codon 6 (A>T); codon 6 (-A); codon 8 (-AA); codon 8/9 (+G); codon 15 (G>A); codon 27 (G>T); IVS I-1 (G>A); IVS I-5 (G>C); IVS I-6 -(T>C); IVS I-110 (G>A); IVS I-116 (T>G), IVS -I-130 (G>C); codon 39 (C>T); codon 44 (-C); IVS II-1 (G>A); IVS II-745 (C>G); IVS II-848 (C>A) (Table 2.2).

Table 2.2: Twenty-two β-globin mutations covered by the StripAssay MED

Position	Sequence alteration	β-thalassemia type
−101	[C>T]	+
−87	[C>G]	+
−30	[T>A]	+
Codon 5	[−CT]	0
Codon 6	[G>A]Hb C	–
Codon 6	[A>T]Hb S	–
Codon 6	[−A]	0
Codon 8	[−AA]	0
Codon 8/9	[+G]	0
Codon 15	[TGG>TGA]	0
Codon 27	[G>T]Hb Knossos	+
IVS 1-1	[G>A]	0
IVS 1-5	[G>C]	+
IVS 1-6	[T>C]	+
IVS 1-110	[G>A]	+
IVS 1-116	[T>G]	0
IVS 1-130	[G>C]	0
Codon 39	[C>T]	0
Codon 44	[−C]	0
IVS 2-1	[G>A]	0
IVS 2-745	[C>G]	+
IVS 2-848	[C>A]	+

The assay procedure includes 3 steps:

2.8.3.1 Genomic DNA Isolation

The blood in K_3-EDTA tubes were used for DNA extraction that was performed either immediately or kept frozen at -20 for later DNA processing by a phenol-chloroform method [197, 198].

Reagents:

1. Red cell lysing solution, 2x lysis buffer (100 ml):
    - Ammonium chloride (0.829 g)
    - Potassium hydrogen carbonate (0.092 g)
    - 0.5 M EDTA at pH 7.5 (0.4 ml)

   Made up with distilled water, autoclaved and store at 4 ºC.
2. Sodium Dodecyl Sulphate (SDS) 10% (10 ml):
    - Sodium Dodecyl Sulphate (Sigma, 1 gm)

   Made up with distilled water (dissolution assisted by heating)
3. WBC lysing solution, Salt/EDTA Buffer (100 ml):
    - Sodium Chloride (0.44 g)
    - 0.5 M EDTA at pH 7.5 (4.8 ml)

   Made up with distilled water, autoclave and store at 4 ºC
4. Proteinase K 20 mg/ml (Promega)
5. Phenol:
    - Crystalline phenol (chromatography grade-BDH), was melted at 68 ºC water bath, and then overlayed with an equal volume of 1 M Tris (pH 8), and was allowed to stand overnight at 4 ºC. The 1 M Tris was then discarded, to be replaced by 0.1 M Tris (pH 8), and stored at 4 ºC for up to 4 weeks.
6. Chloroform/Isoamyl alcohol:

   24 volumes of chloroform are mixed with 1 volume of Isoamyl alcohol.

7. 7.5M Ammonium Acetate:

- 5.782 g Ammonium Acetate in 10 ml distilled water and sterilized by filtration.

8. Ethanol 100%
9. Ethanol 70%

Procedure:

1. One volume of the blood was mixed well with two volumes of 2xLysis buffer, in a sterile stoppered glass tube, by inverting several times and the mixture was left at 4 ^{0}C for 10-30 minutes to lyse the red blood cells.
2. Centrifuged at 3000 rpm for 10 minutes to obtain a nuclear pellet of white blood cells at the bottom of the tube.
3. Remove the supernatant carefully and re-suspend the nuclear pellet in 1 ml Salt/EDTA buffer then vortex briefly.
4. Add 100 µL of 10% SDS to the re-suspended pellet and then 10 µL of Proteinase K (20 mg/ml).
5. Incubated overnight at 37 ^{0}C water bath.
6. Add 1 ml of Phenol (Saturated with 0.1 M Tris, pH 8), and then mix on a rotary mixer, for 10 minutes.
7. Centrifuge at 2000 rpm for 5 minutes.
8. Remove the supernatant to another stoppered glass tube and add another 1 ml of phenol.
9. Remove the supernatant and add 1 ml Chloroform/Isoamyl alcohol (24:1) in another stoppered glass tube.

10. Centrifuge again at 2000 rpm for 5 minutes.
11. Remove the supernatant and add another 1 ml chloroform/Isoamyl alcohol (24:1).
12. Centrifuge at 2000 rpm for 5 minutes
13. Remove the supernatant from this last step to another glass tube and add 0.5 ml of 7.5M Ammonium Acetate and 3 ml of absolute ethanol 100% and gently shake to precipitate DNA.
14. Centrifuge at 2000 rpm for 5 minutes.
15. Remove the supernatant and add 70% ethanol to the pellet. Shake well and centrifuge at 2000 rpm for 5 minutes.
16. Remove the supernatant and leave the DNA pellet stuck to the bottom of the tube. Air-dry the pellet until all the ethanol has evaporated.
17. Dissolve the DNA pellet in 100 μL of sterile water.
18. Leave at 4ºC for 4-5 hours for the DNA to dissolve.
19. The resulting DNA template suitable for immediate use in PCR. For further storage, the DNA solution should be transferred into a sterile eppendorf tube and kept refrigerated (2-8^{o} C; up to one week) or frozen at -20^{o}C for long-term storage.

Genomic DNA Qualification

Following genomic DNA extraction from peripheral blood leucocytes, the DNA samples quality and quantity were checked using ultraviolet spectrophotometry (nanodrop). A DNA concentration range of 2-20 μg/ml (10-100 ng DNA per reaction) is recommended.

2.8.3.2 PCR Amplification Using Biotinylated Primers

Reagents:

All PCR reagents and DNA templates were kept refrigerated throughout.

1. Taq DNA polymerase (5 µl)
2. Taq dilution buffer (500 µl)
3. Amplification mix contain biotinylated primers (500 µl)

PCR Set Up Procedure:

The following PCR reaction was based on a 25 µl reaction volume, and all steps were performed on ice (0-4°C):

1. Prepare a fresh working dilution (0.2 U/µl) of Taq DNA polymerase in Taq Dilution Buffer.
2. Prepare one rection tube for each sample to be amplified, and place the tubes on ice.
3. For each sample prepare a final PCR reaction mix on ice:

- 15 µl amplification mix
- 5 µl diluted Taq DNA polymerase (1U).
- 5 µl DNA template.

4. Capp tubes tightly. Preheating the thermocycler to 94°C.
5. Insert reaction tubes and run the following thermocycling program:

- Pre-PCR: 94°C/2 min.
- Thermocycling: 94° C/15 sec. -58° C/30 sec. -72° C/45 sec. (35 cycles).
- Final extension: 72°C/3min.

Then store the amplification product on ice or at 2-8°C for further use.

2.8.3.3 Hybridization

Reagents

1. Hybridization buffer (25 ml)
2. Wash solution A (80 ml)
3. DNAT (1.5 ml)
4. Conjugate solution (25 ml)
5. Wash solution B (80 ml)
6. Color developer (25 ml)
7. Teststrips (20)
8. Typing trays (3)

Procedure:

1. Adjust the water level of the water-bath to approximately ½ of the height of the Typing Tray.
2. Heat the water-bath to exactly 45° C (± 0.5° C). The water temperature needs to be checked with a calibrated thermometer.
3. Prewarm hybridization buffer and wash solution A to 45°C until all precipitate formed at 2-8°C become completely dissolved.
4. Allow Teststrips, DNAT, conjugate solution, wash solution B and color developer to reach room temperature.
5. Prepare typing tray, then remove one Teststrip for each sample using clean tweezers. Label Teststrips outside of the marker lines with a pencil.
6. Pipette 10 µl DNAT into the lower corner of each lane to be used in the typing trays.

7. Add 10 µl amplification product into the corresponding drop of DNAT. Mix thoroughly with a pipette (the solution will remain blue).
8. Let to stand for 5 min. at room temperature.
    a. Add 1 ml hybridization buffer (prewarmed to 45°C) into each lane. Gently agitate tray (The blue color will disappear).
9. Insert Teststrips with a marked side up (lines visible) into the respective lanes. Submerging completely.
10. Incubate for 30 min. at 45°C on the shaking platform of the water-bath. Set moderate shaking frequency (approx. 50 rpm) to avoid spilling and keeping the cover of the water-bath closed to avoid variations in temperature.
11. At the end of incubation remove hybridization solutions by vacuum aspiration. Do not allow Teststrips to run dry during the entire procedure.

Stringent Wash procedure:

1. Add 1 ml wash solution A (prewarmed to 45°C), then rinse briefly for 10 seconds. Later remove liquids by vacuum aspiration.

2. Add 1 ml wash solution A (45°C).

3. Incubate for 15 minutes at 45°C in the shaking water-bath, then remove liquid by vacuum aspiration.

Color Development procedure:

1. Add 1 ml conjugate solution

2. Incubate for 15 minutes at room temperature on a rocker or orbital shaker, then remove liquids by vacuum aspiration.

3. Add 1 ml wash solution B, rinse briefly for 10 seconds, then remove liquids by vacuum aspiration.

6. Add 1 ml solution B, incubate for 5 minutes at room temperature on a rocker or orbital shaker, then remove liquids by vacuum aspiration.

7. Add 1 ml color developer.

8. Incubate for 15 minutes at room temperature in the dark on a rocker or orbital shaker. A purple staining will appear upon positive reaction.

9. Wash Teststrips several times with distilled water. Then let strips dry in the dark on absorbent paper, and Teststrips should not be allowed to expose to intense light after color development.

2.8.3.A Interpretation of Results

The genotype of a sample is determined using the enclosed Collector™ sheet. Place the processed Teststrip into one of the designated fields, align it to the schematic drawing using the red marker line (top) and the green marker line (bottom), and fix it with adhesive tape.

A positive reaction of the uppermost control line indicated the correct function of conjugate solution and color developer. This line should always stain positive.

For each polymorphic position, one of the following staining patterns should be obtained (Figure 2.2), (Table 2.3):

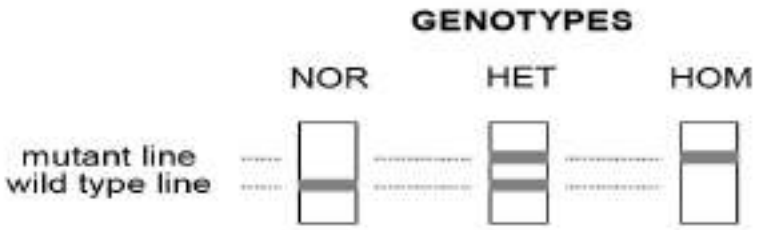

Figure 2.2: Genotype results interpretation.

Table 2.3: Genotype results in normal, heterozygous and homozygous mutation states.

	wild type line	mutant line	genotype
NOR	**positive**	negative	normal
HET	**positive**	**positive**	heterozygous
HOM	negative	**positive**	homozygous mutant

Some of the mutations covered by the β-Globin StripAssay ® MED are located within a few nucleotides on the β-globin gene. On the Teststrips these are represented by a common wild type probe, so that the 22 mutations are covered by 13 wild type probes only (Table 2.4).

Table 2.4: Twenty-two mutations with their wild type probes.

line	wild type probe	mutation
23	- 101 to - 87	- 101, - 87
24	- 30	- 30
25	codon 5 to 9	codon 5, HbC, HbS, codon 6, codon 8, codon 8/9
26	codon 15	codon 15
27	codon 27	codon 27
28	IVS 1.1 to IVS 1.6	IVS 1.1, IVS 1.5, IVS 1.6
29	IVS 1.110 to IVS 1.116	IVS 1.110, IVS 1.116
30	IVS 1.130	IVS 1.130
31	codon 39	codon 39
32	codon 44	codon 44
33	IVS 2.1	IVS 2.1
34	IVS 2.745	IVS 2.745
35	IVS 2.848	IVS 2.848

Some examples of StripAssay results are shown in Figure 2.3.

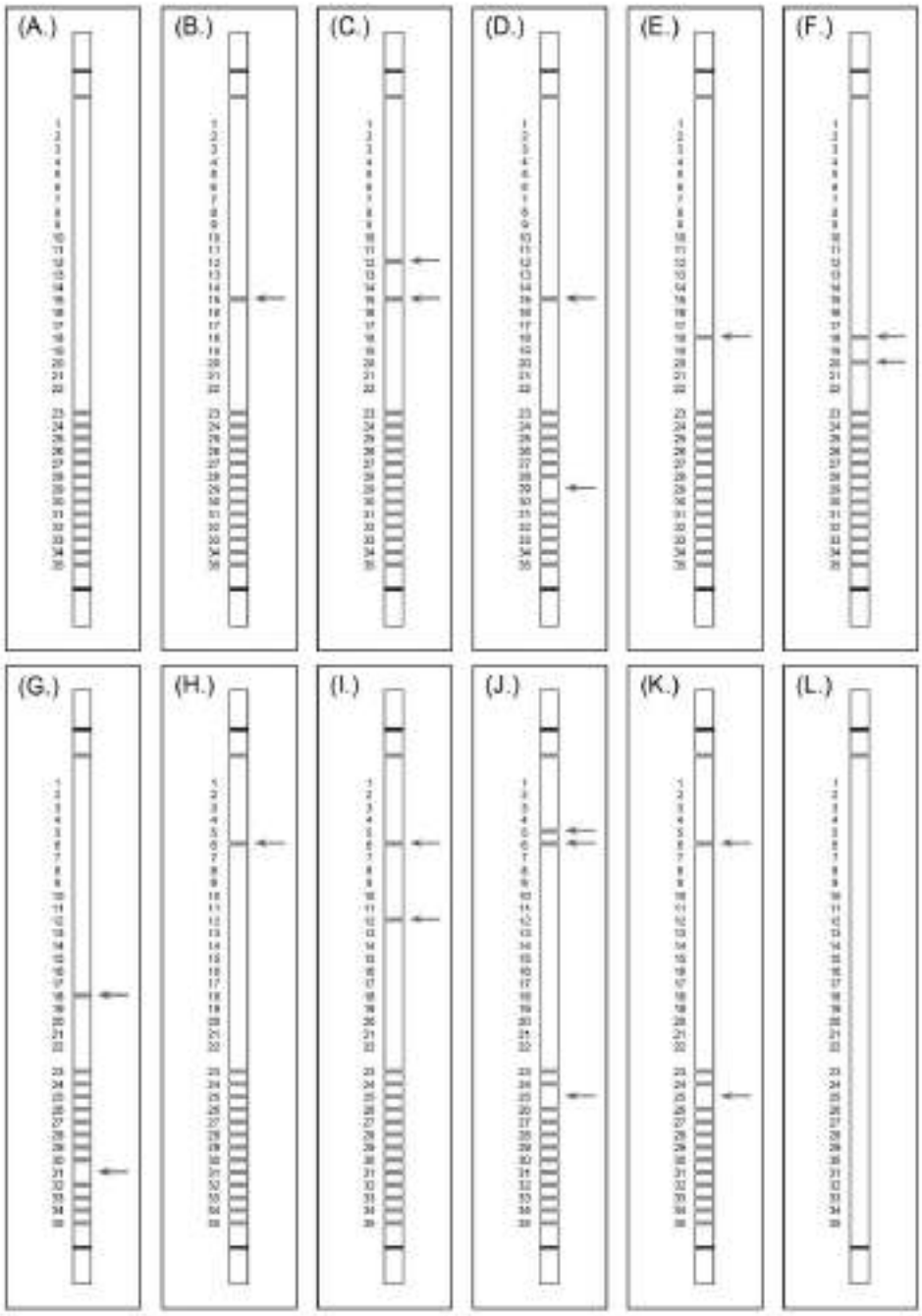

Figure 2.3: Examples of StripAssay results.

2.8.3.B DNA Sequencing

Direct sequencing of the entire β-globin gene (*HBB*) was performed for 24 patients when reverse hybridization did not reveal the underlying β-globin alleles (or only one mutant allele was detected) at Kariminejad-Najmabadi Pathology and Genetic center in Tehran, Iran using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI Prism 377 DNA Automatic sequencer (Perkin Elmer, Foster City, CA). Screening for -α $^{3.7}$, -α $^{4.2}$, and the $–^{Med-1}$ α-thal deletions and α-gene triplication by gap PCR [199] was also conducted for the above 24 uncharacterized samples.

2.9 Statistical Analysis

SPSS program (version 21) was applied for data analysis, (IBM SPSS Statistical Package for the Social Sciences). Means and SD were calculated in continuous data and the frequency proportion for categorical data. For quantitative variables, the difference in the means was assessed using an independent t-test and while comparing the means among several groups was performed using the ANOVA test. A Chi-square test was used to compare the categorical data among different groups of patients. Multivariate logistic regression analysis was used to estimate the independent effect of different study variables on disease-related complications. P -values of < 0.05 were used as a cutoff point for the significance of statistical tests.

CHAPTER THREE

Results

3.1 All β-Thalassemia Patients

3.1.1 Demographic and Clinical Characteristics of All β-Thalassemia Patients

A total of 242 β-thal patients from 162 families were recruited; including 159 thal intermedia (TI) and 83 thal major (TM) patients. The mean age was 17 ± 10.2 years, with a range of (1.4-54 years). They were 129 males (53.3%) and 113 females (46.7%), with a male: female ratio (1.1:1). The mean age at diagnosis and initiation of transfusion was 5 ± 6.3 years and 4 ± 5.8 years, respectively. One hundred and twenty-three (50.8%) patients had splenomegaly, with a mean spleen size of 13.8 ± 3.5cm, 34.3% of which underwent splenectomy. On the other hand, 87 thalassemia patients (36%) had hepatomegaly, with a mean hepatic size of 13.8 ± 2.5cm (Table 3.1).

Table 3.1: Demographic and disease characteristics of 242 β-thalassemia patients.

Parameter	No. (%)
Age (years)	
• <18	144 (59.5)
• 18-34	82 (33.9)
• ≥35	16 (6.6)
Splenomegaly	123 (50.8)
Mean spleen size ± SD	13.8 ± 3.5
Splenectomized	83 (34.3)
Hepatomegaly	87 (36.0)
Mean liver size ± SD	13.8 ± 2.5
Serum ferritin (μg/dL)	
• <1000	148 (61.2)
• ≥1000	94 (38.8)
Treatment	
• None transfused	33 (13.6)
• Occasional transfusion	77 (31.8)
• Regular transfusion	132 (54.5)
• Iron chelation	142 (58.7)
• Hydroxyurea	84 (34.7)
Complications	
• Bone disease	162 (66.9)
▲ Facial deformity	162 (66.9)
▲ Osteoporosis	37 (37)
• Endocrinopathies	78 (32.2)
▲ Growth retardation	49 (34)
▲ Hypothyroidism	33 (15.5)
▲ Diabetes mellitus	5 (2.1)
• Hepatobiliary disease	70 (28.9)
▲ High ALT ≥50 IU/l	31 (12.9)
▲ Biliary complications	48 (19.9)
• Pulmonary hypertension	24 (9.9)
• Thrombosis	2 (0.8)
• EMH	1 (0.4)
• Leg ulcer	1 (0.4)
Rate of complications	191 (78.9)

Osteoporosis and hypothyroidism were evaluated in patients ≥10 years old and/or symptomatic [10], and growth retardation (height >2 SD below 3rd percentile for the mean age and gender) was evaluated in patients ≤18 years [188, 189].

3.1.2 Conventional Management of All β-Thalassemia Patients

As for thalassemia patients' management, 132 patients (54.5%) were on regular transfusion therapy (>3 times /year), while 77 (31.8%) patients received occasional transfusion regimen (0-3/year); during a severe illness, pregnancy, or surgery, whereas 13.6% had never been transfused. In addition, 142 patients (58.7%) received iron chelating therapy for at least one-year duration, and Deferasirox and Desferoxamine were used in 83.9% and 16.1%, respectively. In the current study, 84 (34.7%) were using hydroxyurea therapy and had revealed a potentially lower annual transfusion rate (3 ± 5.3), a lower mean S. ferritin (695.2 ± 1039.5 µg/l) and hence, a significantly lower requirement for iron chelation therapy, in 18.3% as opposed to those patients without hydroxyurea therapy, *p*-value <0.001 (Table 3.2).

Table 3.2: Impact of hydroxyurea therapy on disease characteristics.

Hydroxyurea Therapy (N, %)	Transfusion/year (mean ± SD)	S. ferritin (mean ± SD)	Chelation Therapy (N, %)
• Yes 84 (34.7)	3 ± 5.3	695.2 ± 1039.5	26 (18.3)
• No 158 (65.3)	10.4 ± 6.5	1553.4 ± 1584.5	116 (81.7)
	P value <0.001	*P* value <0.001	*P* value <0.001

3.1.3 Disease Associated Morbidities

Bone disease was the most recurrent morbidity, reported in 162 patients (66.9%), including facial deformity and osteoporosis in (66.9%, and 37%, respectively), followed by endocrinopathies in 78 (32.2%) patients; growth retardation, hypothyroidism, and DM in (34%, 15.5% and 2.1%, respectively), hepatobiliary complications in 70 (28.9%) patients; abnormal

liver function test (LFT); ALT ≥50 IU/l and biliary complications (cholelithiasis and cholecystectomy) in (12.9%, and 19.9%, respectively), and pulmonary hypertension (PHT) in 9.9% (Table 3.1). Other complications; thrombotic events, leg ulcers and extra-medullary hemopoiesis (EMH) were less frequent. No patient with heart failure was identified.

Furthermore, osteoporosis and abnormal LFT were less frequently observed among patients used hydroxyurea therapy, on the other hand, high ALT ≥50 IU/l was more frequently reported among patients with ferritin level ≥1000 µg/l, patients on regular transfusion, and iron chelation therapy, *p*-value <0.001. Moreover, pulmonary hypertension and biliary complications were significantly reported among splenectomized patients, *p*-value <0.05. Additionally, the later complication was significantly documented among patients on occasional transfusion therapy and patients ≥18 years old, *p*-value 0.001 (Table 3.3).

3.3: Univariate analysis of disease-related morbidities among 242 β-thalassemia patients.

	Hypothyroidism (n= 33)	Osteoporosis (n= 37)	Biliary disease (n= 48)	Abnormal LFT (n= 31)	Pulmonary Hypertension (n= 24)
Age (Years)					
< 18 years (n= 144)	19	16	8	19	11
18 - 34 years (n = 82)	13	18	32	11	11
≥ 35 years (n= 16)	1	3	8	1	2
P value	0.62	0.66	< 0.001	0.78	0.32
Gender					
Male (n= 129)	16	23	20	19	12
Female (n= 113)	17	14	28	12	12
P value	0.6	0.11	0.08	0.31	0.76
Ferritin level (µg/l)					
< 1000 (n = 148)	24	23	34	2	12
≥1000 (n = 94)	9	14	14	29	12
P value	0.15	0.69	0.11	< 0.001	0.2
Hemoglobin level (g/dL)					
< 9 (n = 142)	20	27	16	21	15
≥ 9 (n = 97)	13	10	32	10	9
P value	0.76	0.055	< 0.001	0.33	0.74
Splenectomy					
Yes (n = 83)	6	23	38	14	13
No (n = 159)	27	14	10	17	11
P value	0.03	0.78	< 0.001	0.2	0.03
Transfusion/Year					
None (n = 33)	2	2	4	0	1
Occasional (n = 77)	16	9	29	4	9
Regular (n = 132)	15	26	15	27	14
P value	0.07	0.4	< 0.001	< 0.001	0.32
Iron chelation (n=242)					
No chelation (n = 100)	15	6	18	1	4
Yes chelation (n = 142)	18	31	30	30	20
P value	0.17	0.58	0.59	< 0.001	0.01
Hydroxyurea					
Yes (n = 84)	15	6	22	5	10
No (n= 158)	18	31	26	26	14
P value	0.19	0.01	0.06	0.01	0.49

3.1.4 Laboratory Investigations

Hemoglobin level at the time of the study ranged between 4.1-13.8 g/dl with a mean of 8.8 ± 1.3 g/dl. The mean MCV and MCH were (74.4 ± 9.7 fl and 25.6 ± 3.8 pg, respectively). Hb F at first presentation ranged between 4.6% -99.8% with a mean of 65.7 ± 34.8%, while mean Hb A_2 was 3.2 ± 2.3% with a range of 0.2% – 11.2% (Table 3.4).

Table 3.4: Laboratory investigations of all β-thalassemia patients in the current study.

Laboratory Tests	Total
Hb (g/d)	8.8 ± 1.3 (4.1-13.8)
MCV (fl)	74.4 ± 9.7 (48.7-116.2)
MCH (pg)	25.6 ± 3.8 (15.8-38.2)
Hb F (%)	65.7 ± 34.8 (4.6-99.8)
Hb A_2 (%)	3.2 ± 2.3 (0.2-11.2)
ALT (IU/l)	28.3 ± 28.4 (6.0-198)
Serum Ferritin (µg/l)	1250.5 ± 1472.5 (27-9882)
TSH (µIU/ml)	3.5 ± 2 (0.4-15.3)
Free T4 (ng/dl)	18.3 ± 2.9 (14-27.6)
HCV (n, %)	40 (16.5)
HBV (n, %)	0 (0)
HIV (n, %)	0 (0)

The S. ferritin level ranged between 27- 9882 µg/l with a mean of 1250.5 ± 1472.5µg/l. Moreover, 31 patients (12.9%) had been diagnosed with abnormal LFT (ALT ≥50 IU/l) with a mean of 28.3 ± 28.4 IU/l. Over 90% of them had S. ferritin ≥ 1000 µg/l and received iron chelation therapy, 12 (38.7%) had hepatomegaly and 6 (19.4%) were HCV positive. On the other

hand, 75 (31%) patients were reported to have hepatomegaly with normal liver function test (ALT <50 IU/L), almost half of them (56%) used iron chelation therapy, 25.3%, and 22.7% have HCV infection and ferritin level ≥1000 µg/L, respectively (Table 3.5). The mean TSH was 3.5 ± 2µg/l, with a range of 0.4-15.3 µg/, and 33 (15.5%) patients have high TSH, almost all of them 32 (97.0%) were subclinical hypothyroidism presented with high TSH and normal free T4, while just one patient have overt hypothyroidism presented with high TSH and low free T4. Lastly, HCV infection detected in 40 (16.5%) patients (Table 3.4).

Table 3.5: Patients with hepatomegaly and normal liver function test.

Parameter	TI (55, 73.3%)	TM (20, 26.7%)	Total (75, 31%)
Ferritin ≥1000 µg/L	8 (14.5)	9 (45)	17 (22.7)
Iron chelation	23 (41.8)	19 (95)	42 (56)
Type of chelation • Deferasirox • Deferoxamine	 19 (82.6) 4 (17.4)	 19 (100) 0 (0)	 38 (90.5) 4 (9.5)
HCV infection	12 (21.8)	7 (35)	19 (25.3)

3.1.5 Molecular Investigations

Among 484 alleles from 242 β-thalassemia patients, a total of 22 β-globin alleles were identified, 12 of which were β^0, 9 were β^+, with a single dominant β-thalassemia like mutation codon 127 (A >G). The three most frequent β-thal mutations were: IVS-II-1 (G >A), followed by IVS-I-6 (T >C), and codon 8/9; 35.7%, 18.0%, and 8.5%, respectively. Other mutations were less frequent (Table 3.6). Fourteen β-thal mutations were determined by reverse hybridization, while the remaining 8 mutations were

identified by direct sequencing: IVS-I-128 (T>G), CAP +1 (A>C), codon 36/37 (-T), codon 25/26 (+T), codon 82/82 (-G), codon 127 (A>G), +20 (C>T)/IVS-II-745 (C>G), and IVS-II-850 (G>T).

Table 3.6: The reported β-Globin gene mutations in 242 thalassemia patients in the current study.

β-Thalassemia mutations	Total
β^+	
1- IVS I.6 (T>C)	87 (18.0)
2- IVS I.110 (G>A)	29 (6.0)
3- IVS I.5 (G>C)	27 (5.6)
4- IVS I.128 (T>G)	10 (2.0)
5- IVS II.745 (C>G)	8 (1.7)
6- CAP +1 (A>C)	2 (0.4)
7- -101 (C>T)	1 (0.2)
8- -30 (T>A)	1 (0.2)
9- +20 (C>T)	1 (0.2)
Total	166 (34.3)
β^0	
1- IVS II.1 (G>A)	173 (35.7)
2- Cod 8/9 (+G)	41 (8.5)
3- IVS I.1 (G>A)	27 (5.6)
4- Codon 8 (-AA)	26 (5.4)
5- Cod 5 (-CT)	19 (3.9)
6- Cod 39 (C>T)	8 (1.7)
7- Cod 44 (-C)	8 (1.7)
8- Cod 36/37 (-T)	4 (0.8)
9- Cod 25/26 (+T)	2 (0.4)
10- Cod 82-83 (-G)	2 (0.4)
11- Cod 15 (G>A)	1 (0.2)
12- IVS II.850 (G>T)	1 (0.2)
Total	312 (64.5)
Wild	5 (1.0)
Dominant like β-thalassemia Cod 127 (A>G)	1 (0.2)
Total 22 alleles	**484**

In the current study, 53 various genotypes were identified; homozygous IVS-II-1 (24.8%), homozygous IVS-I-6 (13.2%), and compound heterozygous IVS-II-1/codon 8/9 (4.1%) were the most frequent (Table 3.7). Homozygous mutations were determined in (76.3%) patients, of which, 62.9% were the result of the consanguineous marriage, *p*- value <0.001, and parent consanguinity rate was determined in 52.5%.

Table 3.7: Genotypes of 242 thalassemia patients in the current study.

Genotypes	Total
β^0/β^0	
1. IVS II.1 / IVS II.1	60 (24.8)
2. IVS II.1 / Cod 8/9	10 (4.1)
3. Cod 8/9 / Cod 8/9	6 (2.5)
4. IVS I.1 / IVS I.1	6 (2.5)
5. Cod 5 / Cod 5	5 (2.1)
6. Cod 8 / Cod 8	5 (2.1)
7. Cod 8 / IVS II.1	5 (2.1)
8. IVS I.1 / IVS II.1	5 (2.1)
9. Cod 44 / Cod 44	4 (1.7)
10. Cod 8/ Cod 8/9	4 (1.7)
11. IVSI.1 / Cod 8/9	3 (1.2)
12. Cod 39 / Cod 39	2 (0.8)
13. Cod 39 / IVS II.1	2 (0.8)
14. Cod 5 / IVS II.1	2 (0.8)
15. Cod 8 / IVS I.1	2 (0.8)
16. IVS II.1 / Cod 36/37	2 (0.8)
17. Cod 36/37 / Cod 36/37	1 (0.4)
18. Cod 5 / Cod 82-82	1 (0.4)
19. Cod 5 / Cod 8/9	1 (0.4)
20. IVS II.1 / Cod 15	1 (0.4)
21. IVS II.1 / Cod 82-83	1 (0.4)
Total	**128 (52.9)**
β^+/β^+	
1- IVS I.6 / IVS I.6	32 (13.2)
2- IVS I.110 / IVS I.110	6 (2.5)
3- IVS I.5 / IVS I.5	5 (2.1)
4- IVS I.5 / IVS I.6	4 (1.7)
5- IVS II.745 / IVS II.745	3 (1.2)
6- IVS I.128 / IVS I.128	2 (0.8)
7- IVS I.128 / IVS I.110	2 (0.8)
8- IVS I.6 / IVS II.745	1 (0.4)
Total	**55 (22.7)**
β^0/β^+	
1. IVS II.1 / IVS I.6	9 (3.7)
2. IVS II.1 / IVS I.110	7 (2.9)
3. IVS II.1 / IVS I.5	6 (2.5)
4. Cod 8/9 / IVS I.110	5 (2.1)
5. Cod 8/9 / IVS I.5	3 (1.2)
6. Cod 5 / IVS I.128	2 (0.8)
7. Cod 5 / IVS I.6	2 (0.8)
8. Cod 8 / IVS I.110	2 (0.8)
9. IVS I.1 / CAP +1	2 (0.8)
10. IVS I.1 / IVS I.6	2 (0.8)
11. Cod 39 / IVS I.6	2 (0.8)
12. Cod 8 / IVS I.5	2 (0.8)
13. Cod 25/26 / IVS I.5	2 (0.8)
14. IVS I.1 / -101	1 (0.4)
15. Cod 5 / IVS I.110	1 (0.4)
16. Cod 8 / IVS I.6	1 (0.4)
17. Cod 8/9 / - 30	1 (0.4)
18. Cod 8/9 / IVS I.128	1 (0.4)
19. Cod 8/9 / IVS I.6	1 (0.4)
20. IVS II.850 / IVS I.6	1 (0.4)
21. IVS II.1 / IVS I.128	1 (0.4)
Total	**54 (22.3)**
β+/wt +20, IVS II.745 / wt	2 (0.8)
β^0/wt IVS II.1 / wt ($\alpha\alpha^{anti3.7}$)	2(0.8)
Dominant like β-thalassemia Cod 127/ wt	1(0.4)
Total 53 genotypes	**242**

3.1.6 Clinical Characteristics of the Studied Genotypes

Patients with $\beta^0\beta^0$ and $\beta^0\beta^+$ genotypes were diagnosed earlier and had an earlier onset of blood transfusion in comparison to $\beta^+\beta^+$ genotype. Their Hb A_2 level was the lowest and Hb F was the highest. Generally, the above genotypes had a higher frequency of disease morbidities, though not at significant levels when compared with $\beta^+\beta^+$ genotype (apart from facial deformity) (Table 3.8).

Table 3.8: Genotype correlation in 242 thalassemia patients with different parameters.

	β^+ / β^+ (n=55)	β^+/β^0 (n=54))	β^0/β^0 (n=128)	P value
Age at diagnosis (Years)				
Mean ± SD	6.14 ± 8.9	3.02 ± 2.9	4.88 ± 5.4	0.03
Age at first transfusion				
Mean ± SD	4.94 ± 9.2	2.61 ± 2.8	3.67 ± 4.2	0.12
Transfusion/year				
• No	16.4	9.3	14.8	0.51
• Yes	83.6	90.7	85.2	
Iron Chelation				
• Yes	49.1	64.8	60.2	0.22
• No	50.9	35.2	39.8	
HbA_2 %				
• < 3.5	10%	72.7	93.4	< 0.001
• ≥ 3.5	90%	27.3	6.6	
Hb F %				
• < 50.0	90%	13.6	9.5	< 0.001
• ≥ 50	10%	86.4	90.5	
Splenectomy				
• Yes	35.2	33.3	33.9	0.98
• No	64.8	66.7	66.1	
PHT				
• Yes	3.7	14.8	10.4	0.15
• No	96.3	85.2	89.6	
Hepatomegaly				
• Yes	31.5	31.5	43.2	0.18
• No	68.5	68.5	56.8	
Hepatitis C infection				
• Yes	16.7	11.1	19.2	0.41
• No	83.3	88.9	80.8	
Osteoporosis				
• Yes	38.1	32	39.2	0.82
• No	61.9	68	60.8	
Biliary complications				
• Yes	24.1	9.3	20	0.11
• No	75.9	90.7	80	
Hypothyroidism				
• No	84.9	83.0	86.1	0.87
• Yes	15.1	17.0	13.9	
Growth retardation				
• Yes	45.2	22.5	43.9	0.05
• No	54.8	77.5	56.1	
ALT				
• ALT ≥50	7.5	11.1	16.9	0.21
• ALT <50	92.5	88.9	83.1	
Ferritin level				
• < 1000	68.5	57.4	60.8	0.47
• ≥ 1000	31.5	42.6	39.2	
Facial deformity				
• Yes	54.5	64.8	73.4	0.04
• No	45.5	35.2	26.6	

Osteoporosis and hypothyroidism were evaluated in patients ≥10 years old and/or symptomatic, and growth retardation (height >2 SD below 3rd percentile for the mean age and gender) was evaluated in patients ≤18 year.

3.2 β-Thalassemia Intermedia

3.2.1 Demographic and Patient's Characteristics

One hundred and fifty-nine β-thalassemia intermedia (β-TI) patients were enrolled, including 90 (56.6%) males and 69 (43.4%) females, with a male: female ratio of 1.3:1. The patients' ages ranged between 1.4 and 54 years with a median of 15yrs. Age at diagnosis varied between 1 and 50yrs with a median of 5yrs. Ninety-three patients (58.5%) had splenomegaly, of which, 32.7% had splenectomy, while hepatomegaly and HCV were reported in 37.7% and 11.3%, respectively (Table 3.9).

Table 3.9: Patient and disease characteristics of 159 β-TI in the current study.

Parameter	Frequency	Number of evaluated patients	Percent
Demographic Data			
Age (years)			
• <18	90	159	56.6
• 18-35	55	159	34.6
• >35	14	159	8.8
Gender			
• Male	90	159	56.6
• Female	69	159	43.4
Splenomegaly	93	159	58.5
Splenectomized	52	159	32.7
Hepatomegaly	60	159	37.7
Serum Ferritin (µg/L)			
• <1000	122	159	76.7
• ≥1000	37	159	23.3
Treatment			
• None Transfused	33	159	20.7
• Occasional Transfusion	75	159	47.2
• Regular Transfusion	51	159	32.1
• Iron Chelation	63	159	39.6
• Hydroxyurea	75	159	47.2
Complications			
Facial Deformity	99	159	62.3
*Osteoporosis	17	60	28.3
**Growth Retardation	25	90	27.8
***Subclinical Hypothyroidism	22	131	16.8
**** Cholelithiasis	19	137	13.8
Pulmonary Hypertension	18	159	11.3
Abnormal liver function	12	159	7.5
Thrombosis	2	159	1.3
EMH	1	159	0.6
Leg Ulcer	1	159	0.6
Rate of complications	122	159	76.7

*Osteoporosis and ***subclinical hypothyroidism were evaluated in patients ≥10 years old and/or symptomatic [10],** growth retardation (height >2SD below 3rd percentile for the mean age and gender) in patients ≤18 years [188, 189], and**** cholelithiasis estimated in 137 patients excluding the 22 patients that underwent cholecystectomy.

3.2.2 Transfusion History, Chelation and Hydroxyurea Therapy

The median age at onset of transfusion was 4.7 years with a range between 1 and 50 yrs. Almost half of the patients (47.2%) received occasional transfusion sessions ranging from 0 to 3 per year (during severe infection, operation, or pregnancy), while just 32.1% of the patients received regular transfusion >3/year, whereas 20.7% had never been transfused. Sixty-three patients (39.6%) received iron-chelating drugs for at least a one-year duration. Deferasirox was used in 85.7%, and deferoxamine was used in 14.3%, while 60.3% of the β-TI patients did not receive any type of chelating drugs. Hydroxyurea, on the other hand, was given to 47.2% of the patients (Table 3.9).

3.2.3 Disease-associated Morbidities

The most common disease-associated complication was bone disease; facial deformity (62.3%), and osteoporosis (28.3%). Endocrinopathies were second, including [growth retardation (27.8%), and subclinical hypothyroidism (16.8%)]. Cholelithiasis followed at (13.8%), PHT (11.3%), and abnormal liver function (7.5%) (Table 3.9). Thrombosis, EMH, and leg ulcers were less frequent, while diabetes mellitus and heart failure were not identified. Furthermore, the probability of developing the above morbidities increased significantly with age (Figure 3.1).

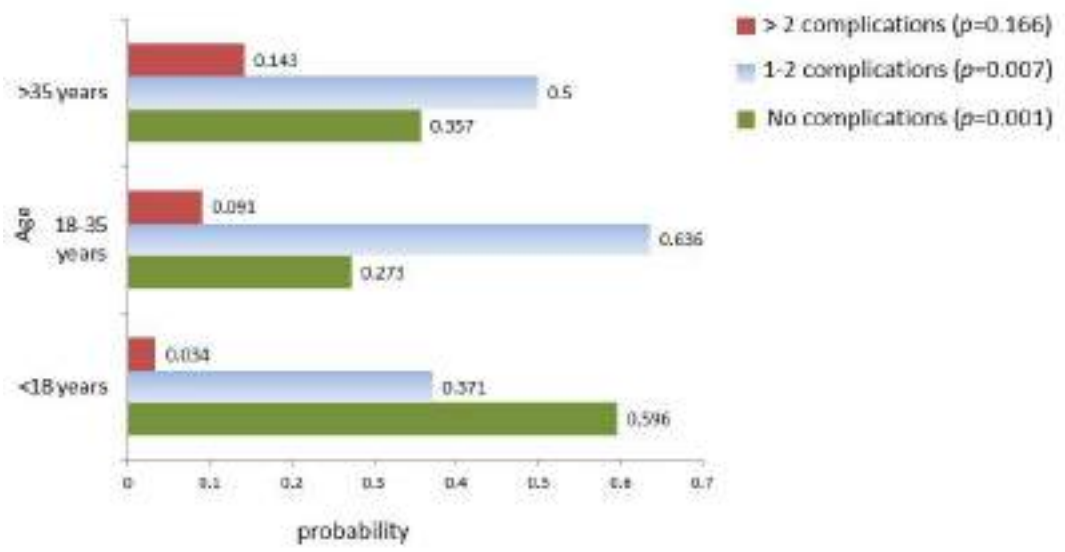

Figure 3.1: Probability of developing disease-related morbidities among 159 β-TI patients at different age intervals.

3.2.4 Multivariate Analysis

Selected variables were studied in a logistic regression analysis to identify their role in the pathophysiology of disease-related complications; PHT, cholelithiasis, hypothyroidism, and osteoporosis (Table 3.10). Age ≥35 was an independent risk factor for cholelithiasis and hypothyroidism. Likewise, female sex was associated with an increased risk of cholelithiasis and osteoporosis. Whereas mean serum ferritin of ≥1000 μg/L was independently associated with an increased risk of osteoporosis, iron chelation therapy was protective for a multitude of other complications (PHT, cholelithiasis, hypothyroidism, and osteoporosis). Although transfusion was associated with an increased risk of osteoporosis, it was protective for cholelithiasis and hypothyroidism. Moreover, splenectomy was protective for cholelithiasis, while it was an independent risk for hypothyroidism. Finally, hydroxyurea was associated independently with increased risk of osteoporosis, though it was protective for cholelithiasis.

Table 3.10: Multivariate analysis for determinants of complication rate in 159 β-TI patients.

	RR	95 % CI	*P* value
Pulmonary Hypertension			
Age ≥ 35 years	0.798	(0.13 - 4.64)	0.80
Splenectomy	0.839	(0.27 - 2.6)	0.76
Transfusion	0.670	(0.06 - 6.81)	0.74
Hydroxyurea	0.600	(0.19 - 1.82)	0.37
Iron chelation	0.160	(0.03 - 0.65)	0.01
Cholelithiasis			
Age ≥ 35 years	1.53	(0.19 - 6.73)	0.87
Gender (Female)	1.693	(0.53 - 5.34)	0.37
Splenectomy	0.140	(0.03 - 0.50)	0.003
Transfusion	0.537	(0.05 - 5.27)	0.59
Hydroxyurea	0.179	(0.05 - 0.60)	0.006
Iron chelation	0.325	(0.08 - 1.19)	0.09
Hypothyroidism			
Age ≥ 35 years	1.817	(0.20 - 16.14)	0.59
Splenectomy	2.178	(0.70 - 6.75)	0.18
Transfusion	0.251	(0.05 - 1.24)	0.09
Hydroxyurea	0.645	(0.23 - 1.79)	0.40
Iron chelation	0.552	(0.241 - 1.265)	0.15
Osteoporosis			
Age ≥ 35 years	0.616	(0.235- 1.62)	0.36
Gender (Female)	4.139	(0.87 - 19.58)	0.07
Ferritin ≥ 1000 μg/L	6.86	(1.09 - 42.97)	0.04
Splenectomy	0.994	(0.24 - 4.11)	0.99
Transfusion	14.352	(0.86 - 221.12)	0.06
Hydroxyurea	9.004	(1.67 - 48.41)	0.01
Iron chelation	0.443	(0.09 - 2.08)	0.30

3.2.5 Laboratory Investigations

The mean Hb at the time of enrollment was 8.9 ± 1.4 g/dL, with a range of 4.1-13.8 g/dL. The mean MCV and MCH were 72 ± 10.6 and 24.4 ± 4, respectively. The Hb F level at the time of the first presentation ranged between 4.6 and 99.5% with a mean of 65.7 ± 34.8%, while Hb A_2 ranged from 0.4 to 8.4%, with a mean of 3.2 ± 2.2%. Furthermore, the serum ferritin level ranged from 27 to 9882 μg/L with a mean of 853.3 ± 1192.7 μg/L (Table 3.11). One hundred and twenty-two patients (76.7%) had a serum ferritin level <1000 μg/L, while just 23.3% had a ferritin level ≥1000 μg/L. Elevated ALT ≥50 IU/L, on the other hand, was reported in 12 patients, (91.7%, 75%, and 41.7%) of them received iron chelation therapy, had a ferritin level ≥1000 μg/L, and hepatomegaly, respectively), while none was HCV positive. HBV infection was not reported.

Table 3.11: Laboratory investigations of 159 β-TI patients in the current study.

Laboratory tests	Mean ± SD	Range
Hb (g/dL)	8.9 ± 1.4	4.1 – 13.8
PCV (L/L)	26.6 ± 4.4	10.5 – 42.7
MCV (fl)	72 ± 10.6	48.7 – 116.2
MCH (pg)	24.4 ± 4	15.8 – 38.2
Hb A_2 (%)	3.2 ± 2.2	0.4 – 8.4
Hb F (%)	65.7 ± 34.8	4.6 – 99.5
S. Ferritin (µg/L)	853.3 ± 1192.7	27 – 9882
ALT (IU/l)	23.4 ± 23.8	6.0 – 158
TSH (µIU/ml)	3.5 ± 1.9	0.4 – 14.8
Free T4 (Pmol/L)	18.6 ± 2.8	14.7 – 27.6
HCV infection (n, %)	18 (11.3)	------

Regarding thyroid function tests, the mean TSH was 3.5 ± 1.9 µIU/ml, ranged from 0.4-14.8 µIU/ml, with a mean free T4 of 18.6 ± 2.8 ng/dl, and 22 patients (16.8%) were reported to have subclinical hypothyroidism, presented with high TSH >4.7 µIU/ml and normal free T4 level. Lastly HCV infection was determined in 18 (11.3%) patients.

3.2.6 Molecular Investigations

A total of 19 different β-thalassemia mutations were determined among 159 TI patients; 12 (63.2%) were identified by reverse hybridization with IVS-II-I (G>A) (47.2%) as the most prevalent mutation, followed by IVS-I-6 (T>C) (23.3%), and IVS-I-110 (G>A) (5.0%). Other mutations were less prevalent or sporadic (Table 3.12). On the other hand, 7 mutations were detected by direct sequencing: CAP +1 (A>C), IVS-I-128 (T>G), +20 (C>T) /IVS-II.745 (C>G), codon 36-37 (- T), codon 82-83 (-G), IVS-II.850 (G>T), and codon 127 (A>G).

The current work had determined 37genotypes; the most frequent was a homozygous IVS-II-1 (35.9%), followed by a homozygous IVS-I-6 (18.9%), and IVS-II-1 /IVS-I-6 (4.4%) (Table 3.13). Twenty-four families (21.1%) had inherited a homozygous or compound heterozygous β^+/β^+ mutation, while 20.2% had the β^0/β^+ genotype and 56.1% had the β^0/β^0 genotype. Among our patients; 52.8% were the results of a consanguineous marriage. Moreover, those who inherited homozygous mutations were significantly associated with consanguinity (45.3% of patients) with a *P* value < 0.001.

Table 3.12: β-globin gene mutations in 159 thalassemia intermedia patients in the current study.

β-Thalassemia mutations	Frequency of allele	Percent
Very mild β^{++}		
1. CAP+1 (A>C)	2	(0.6)
2. -101 (C>T)	1	(0.3)
Mild β^{+}		
1. IVS I.6 (T>C)	74	(23.3)
2. IVS I.128 (T>G)	8	(2.5)
3. IVS II.745 (C>G)	3	(0.9)
Sever β^{+}		
1. IVS I.110 (G>A)	16	(5)
2. IVS I.5 (G>C)	1	(0.3)
β^{0}		
1. IVS II.1 (G>A)	150	(47.2)
2. IVS I.1 (G>A)	15	(4.7)
3. Cod 8 (-AA)	15	(4.7)
4. Cod 8/9 (+G)	11	(3.5)
5. Cod 5 (-CT)	6	(1.9)
6. Cod 39 (C>T)	3	(0.9)
7. Cod 36/37 (-T)	2	(0.6)
8. Cod 44 (-C)	2	(0.6)
9. Cod 82-83 (-G)	1	(0.3)
10. IVS II.850 (G>T)	1	(0.3)
11. Cod 15 (G>A)	1	(0.3)
Wild	5	(1.6)
Dominant Like β-thalassemia Cod 127 (A>G)	1	(0.3)

Table 3.13: Genotypes of 159 thalassemia intermedia patients in the current study.

Genotypes	Frequency	Percent
β^0/β^0		
1. IVS II.1/ IVS II.1	57	35.9
2. IVS II.1 / Cod 8/9	5	3.2
3. IVS II.1 / IVS I.1	4	2.5
4. IVS II.1 / Cod 8	4	2.5
5. Cod 8/9 / Cod 8	3	1.9
6. Cod 8 / Cod 8	2	1.3
7. IVS I.1 / Cod 8	2	1.3
8. IVS II.1 / Cod 5	2	1.3
9. IVS II.1 / Cod 36/37	2	1.3
10. IVS II.1 / Cod 39	2	1.3
11. IVS I.1 / IVS I.1	2	1.3
12. Cod 5 / Cod 5	1	0.6
13. IVS II.1 / Cod 15	1	0.6
14. IVS II.1 / Cod 82-83	1	0.6
15. Cod 44/ Cod 44	1	0.6
β^+/β^+		
1. IVS I.6 / IVS I.6	30	18.9
2. IVS I.110 / IVS I.110	4	2.5
3. IVS I.128 / IVS I.128	2	1.3
4. IVS I.110 / IVS I.128	1	0.6
5. IVS I.6 / IVS II.745	1	0.6
β^0/β^+		
1. IVS II.1 / IVS I.6	7	4.4
2. IVS II.1 / IVS I.110	4	2.5
3. Cod 5 / IVS I.128	2	1.3
4. IVS I.1 / IVS I.6	2	1.3
5. Cod 8/9 / IVS I.110	2	1.3
6. IVS I.1 / CAP +1	2	1.3
7. Cod 8 / IVS I.6	1	0.6
8. IVS II.1 / IVS I.5	1	0.6
9. Cod 8/9 / IVS I.6	1	0.6
10. Cod 39 / IVS I.6	1	0.6
11. IVS II.850 / IVS I.6	1	0.6
12. Cod 8 / IVS I.110	1	0.6
13. IVS II.1 / IVS I.128	1	0.6
14. IVS I.1 / -101	1	0.6
β^0/wt IVS II.1 /wt ($\alpha\alpha\alpha^{anti3.7)}$	2	1.25
β^+/wt +20, IVS II.745/wt	2	1.25
Dominant like β-thalassemia Cod 127/wt	1	0.6

3.2.7 Clinical Characteristics of the Studied Genotypes

patients with β^0/β^+ and β^0/β^0 genotypes were diagnosed at an earlier age and transfused earlier in comparison to other reported genotypes. Also, Hb F levels were the highest while Hb A2 levels were the lowest in β^0/β^0 patients. Likewise, β^0/β^+ and β^0/β^0 patients showed a higher frequency of PHT in comparison to other genotypes (Table 3.14).

Table 3.14: Relations of different parameters in the all genotype groups in 159 β-TI patients.

Parameters	β^0/β^0 N (%)	β^0/β^+ N (%)	β^+/β^+ N (%)	Dominant Hb Houston/wt	β^+/wt N (%)	β^0/wt N (%)	P value
Age at diagnosis Mean ± SD	6.7 ± 5.6	5.2 ± 2.6	8.6 ±9.7	10	20.0 ±2.8	12.5±3.5	0.02
Age at first transfusion Mean ± SD	5.3 ± 4.4	4.8 ± 2.9	7.6±10.8	8	19.0±2.8	12.5±3.5	0.02
Transfusion							
• No	19 (21.3)	5 (18.5)	9 (23.7)	0 (0.0)	0 (0.0)	0 (0.0)	0.90
• Yes	70 (78.7)	22 (81.5)	29(76.3)	1 (100.0)	2 (100.0)	2 (100.0)	
Iron Chelation							
• No	48 (53.9)	19 (70.4)	28 (73.7)	0 (0.0)	0 (0.0)	1 (50.0)	0.07
• Yes	41 (46.1)	8 (29.6)	10 (26.3)	1 (100.0)	2 (100.0)	1 (50.0)	
Hb A_2 %							
• <3.5	52 (92.9)	14 (73.7)	3 (10.3)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001
• ≥3.5	4 (7.1)	5 (26.3)	26 (89.7)	1 (100.0)	1 (100.0)	2 (100.0)	
Hb F %							
• <50	4 (6.9)	3 (15.8)	26 (89.7)	1 (100.0)	1 (100.0)	2 (100.0)	<0.001
• ≥50	54 (93.1)	16 (84.2)	3 (10.3)	0 (0.0)	0 (0.0)	0 (0.0)	
Splenectomy							
• No	55 (62.5)	21 (77.8)	27 (73.0)	0 (0.0)	0 (0.0)	2 (100.0)	0.07
• Yes	33 (37.5)	6 (22.2)	10 (27.0)	1 (100.0)	2 (100.0)	0 (0.0)	
PHT							
• No	77 (86.5)	23 (85.2)	37 (97.4)	0 (0.00	2 (100.0)	2 (100.0)	0.04
• Yes	12 (13.5)	4 (14.8)	1 (2.6)	1 (100.0)	0 (0.0)	0 (0.0)	
Hepatomegaly							
• No	51 (57.3)	18 (66.7)	27 (71.1)	1 (100.0)	1 (50.0)	1 (50.0)	0.65
• Yes	38 (42.7)	9 (33.3)	11 (28.9)	0 (0.0)	1 (50.0)	1 (50.0)	
Hepatitis C							
• No	77 (86.5)	25 (92.1)	35 (92.1)	0 (0.0)	2 (100.0)	2 (100.0)	0.08
• Yes	12 (13.5)	2 (7.4)	3 (7.9)	1 (100.0)	0 (0.0)	0 (0.0)	
Osteoporosis							
• No	21 (61.8)	10 (90.9)	10 (83.3)	1 (100.0)	1 (50.0)	--	0.26
• Yes	13 (38.2)	1 (9.1)	2 (16.7)	0 (0.0)	1 (50.0)	--	
Cholelithiasis							
• No	78 (87.6)	25 (92.6)	32 (84.2)	1 (100.0)	2 (100.0)	2 (100.0)	0.88
• Yes	11 (12.4)	2 (7.4)	6 (15.8)	0 (0.0)	0 (0.0)	0 (0.0)	
Hypothyroidism							
• No	67 (87.0)	18 (72.0)	31 (86.1)	1 (100.0)	2 (100.0)	2 (100.0)	0.35
• Yes	10 (13.0)	7 (28.0)	5 (13.9)	0 (0.0)	0 (0.0)	0 (0.0)	

Growth retardation							
• No	31 (60.8)	17 (89.5)	16 (84.2)	--	--	1 (100.0)	0.32
• Yes	20 (39.2)	2 (10.5)	3 (15.8)	--	--	0 (0.0)	
ALT							
• ≤50	81 (91.0)	24 (88.9)	37 (97.4)	1 (100.0)	2 (100.0)	2 (100.0)	0.78
• >50	8 (9.0)	3 (11.1)	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	
S. ferritin							
• <1000	65 (74.7)	22 (81.5)	31 (83.)	1 (100.0)	1 (50.0)	2 (100.0)	0.66
• ≥1000	22 (25.3)	5 (18.5)	6 (16.2)	0 (0.0)	1 (50.0)	0 (0.0)	
Facial deformity							
• No	22 (24.7)	13 (48.1)	22 (57.9)	1 (100.0)	0 (0.0)	2 (100.0)	0.001
• Yes	67 (75.3)	14 (51.9)	16 (42.1)	0 (0.0)	2 (100.0)	0 (0.0)	

3.3 Thalassemia Major

3.3.1 Demographic and Patient's Characteristics

Eighty-three thalassemia major (TM) patients were enrolled, including 39 (47%) males and 44 (53%) females, with a male: female ratio of 1:1.1. The mean age was 14.9 ± 6.8 years, with a range of (1.8–36.8 years). Almost two third of the patients were <18 years, with just one patient was >35 years old. Thirty (36.1%) patients had splenomegaly, with a mean spleen size of 12.5 ± 3.3 cm, 37.3% of which underwent splenectomy. Hepatomegaly was reported in 27 (32.5%), with a mean hepatic size of 13.5 ± 2.5 cm (Table 3.15).

Table 3.15: Demographic and disease characteristics of 83 thalassemia major patients.

Parameter	Frequency	Percent
Age (years)		
• <18	54	(65.1)
• 18-34	28	(33.7)
• ≥35	1	(1.2)
Splenomegaly	30	(36.1)
Splenectomized	31	(37.3)
Hepatomegaly	27	(32.5)
Serum ferritin (μg/dL)		
• <1000	26	(31.3%)
• ≥1000	57	(68.7%)
Treatment		
• None transfused	0	(0)
• Occasional transfusion	2	(2.4)
• Regular transfusion	81	(97.6)
• Iron chelation	79	(95.2)
• Hydroxyurea	9	(10.8)
Complications		
• Bone disease	63	(75.9)
▲ Facial deformity	63	(75.9)
▲ Osteoporosis	20	(50)
• Endocrinopathies	34	(41)
▲ Growth retardation	24	(44.4)
▲ Hypothyroidism	11	(13.4)
▲ Diabetes mellitus	5	(6)
• Hepatobiliary disease	24	(28.9)
▲ High ALT ≥50 IU/l	19	(23.2)
▲ Biliary complications	7	(8.5)
• Pulmonary hypertension	6	(7.2)
• Thrombosis	0	(0)
• EMH	0	(0)
• Leg ulcer	0	(0)
Rate of complications	69	(83.1)

Osteoporosis and hypothyroidism were evaluated in patients ≥10 years old and/or symptomatic, and growth retardation (height >2 SD below 3rd percentile for the mean age and gender) was evaluated in patients ≤18 years [188, 189].

3.3.2 Transfusion History, Chelation and Hydroxyurea Therapy

The mean age at initiation of transfusion and diagnosis were 0.75 ± 0.51 months, and 0.82 ± 0.52 months, respectively. Almost all patients received regular transfusion and iron chelation therapy for at least one-year duration (97.6%, and 95.2%, respectively). Deferasirox and Desferoxamine were used in 83.5% and 16.5% patients, respectively. Hydroxyurea therapy, on the other hand, was used in just 9 (10.8%) patients (Table 3.15).

3.3.3 Disease-associated Morbidities

Bone disease was the most recurrent morbidity, reported in 63 patients (75.9%), including facial deformity and osteoporosis in (75.9%, and 50%, respectively), followed by endocrinopathies in 34 (41%) patients; growth retardation, hypothyroidism, and DM in (44.4%, 13.4% and 6.0%, respectively). Hepatobiliary complications were reported in 24 (28.9%) patients; abnormal liver function test (LFT); ALT $\geq$50 IU/l and biliary complications (cholelithiasis and cholecystectomy) in (23.2%, and 8.5%, respectively). The fourth common complication was pulmonary hypertension (PHT) in 7.2% (Table 3.15). Moreover, the probability of developing the above morbidities increased significantly with age (Figure 3.2). Other complications; thrombotic events, leg ulcers and extra-medullary hemopoiesis (EMH) were not detected, as well as, no patient with heart failure was identified.

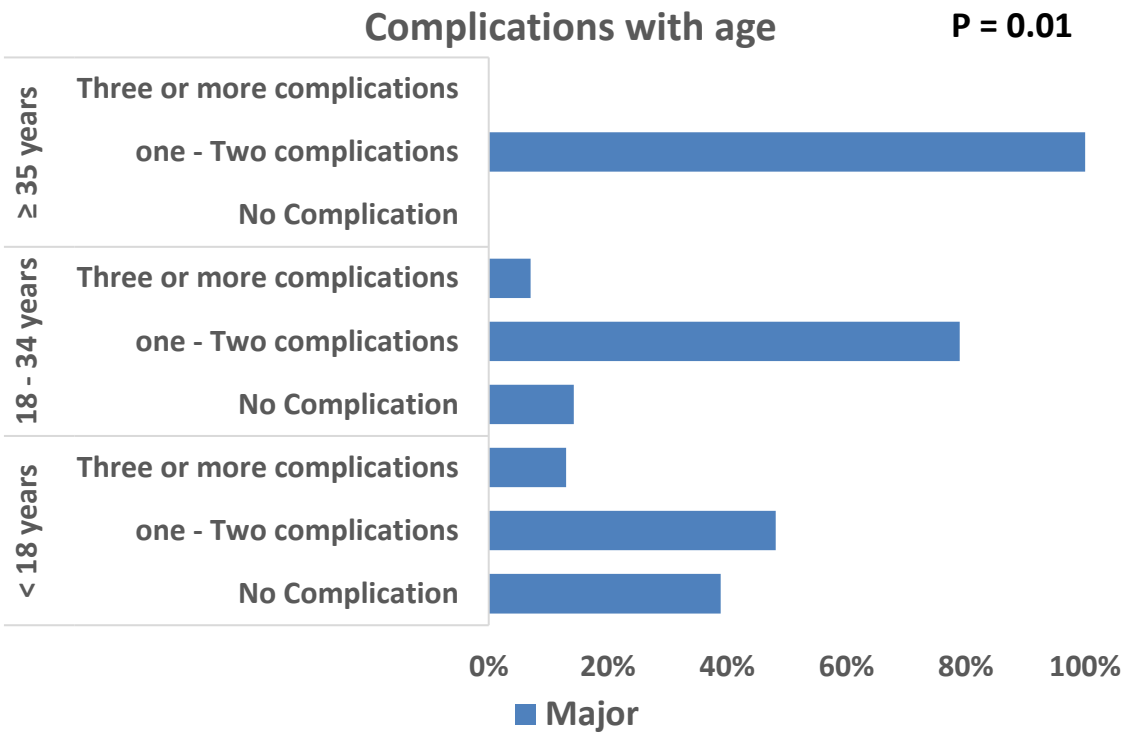

Figure 3.2: Probability of developing disease-related morbidities among 83 thalassemia major patients at different age intervals.

3.3.4 Laboratory Investigations

The mean Hb at the time of enrollment was 8.5 ± 0.9 g/dL, with a range of 5.5-10.8 g/dL. The mean MCV and MCH were 78.9 ± 5.1fL and 27.8 ± 1.9 pg, respectively. The Hb F level at the time of first presentation ranged between 6.2 and 99.8% with a mean of 64.4 ± 35.8%, while Hb A_2 ranged from 0.2 to 11.2%, with a mean of 3.2 ± 3.2%. Furthermore, the serum ferritin level ranged from 300 to 9391 µg/L with a mean of 2006.2 ± 1665.3 µg/L (Table 3.16). Fifty-seven (68.7%) patients had serum ferritin level ≥1000 µg/L, while 26 (31.3%) patients had a ferritin level <1000 µg/L.

Table 3.16: Laboratory investigations of 83 TM patients in the current study.

Laboratory tests	Mean ± SD	Range
Hb (g/dL)	8.5 ± 0.9	5.5 – 10.8
PCV (L/L)	24.7 ± 3.0	17.0 – 33.6
MCV (fl)	78.9 ± 5.1	62.5 – 96.4
MCH (pg)	27.8 ± 1.9	21.4 – 33.6
Hb A_2 (%)	3.2 ± 3.2	0.2 – 11.2
Hb F (%)	64.4 ± 35.8	6.2 – 99.8
S. Ferritin (µg/L)	2006.2 ± 1665.3	300 – 9391
ALT (IU/l)	37.5 ± 33.8	9.0 – 198
TSH (µIU/ml)	3.5 ± 2.1	1.2 – 15.3
Free T4 (Pmol/L)	17.0 ± 3.1	14 – 22.9
HCV infection (n, %)	22 (26.8)	---

Elevated ALT ≥50 IU/L, on the other hand, was reported in 19 patients (23.2%), with a mean of (37.5 ± 33.8 IU/l), and almost all of them had a ferritin level ≥1000 µg/L, were using iron chelation therapy (94.7% and 100%, respectively), and around 1/3rd of them had HCV infection and hepatomegaly (31.6%, and 36.8%, respectively) (Table 3.17). The mean TSH and free T4 were (3.5 ±2.1 µIU/ml and 17.0 ±3.1 ng/dl, respectively). Twenty-two (26.8%) patients were reported to have HCV infection, HBV infection was not reported.

Table 3.17: Elevated liver function test in 19 TM patients with other parameters.

Parameters	Frequency	Percent
• Ferritin ≥1000 μg/L	18	94.7
• Chelation therapy	19	100
• HCV infection	6	31.6
• Hepatomegaly	7	36.8

3.3.5 Molecular Investigations

Among 166 alleles from 83 TM patients, a total of 16 β-globin alleles were identified, 10 of which were β^0, and 6 were β^+. The three most frequent β-thal mutations were: codon 8/9 (+G), followed by IVS I-5 (G>C), and IVS II-1 (G>A); 18.1%, 15.7%, and 13.9%, respectively. Other mutations were less frequent (Table 3.18). Twelve β-thal mutations were determined by reverse hybridization, while the remaining 4 mutations were identified by direct sequencing: IVS-I-128 (T>G), codon 36/37 (-T), codon 25/26 (+T), and codon 82/83 (-G).

Table 3.18: β-globin gene mutations in 83 thalassemia major patients in the current study.

β-Thalassemia mutations	Frequency of allele	Percent
β^+		
1. IVS I-5 (G>C)	26	15.7
2. IVS I.6 (T>C)	13	7.8
3. IVS I-110 (G>A)	13	7.8
4. IVS II-745 (C>G)	6	3.7
5. IVS I.128 (T>G)	2	1.2
6. -30 (T>A)	1	0.6
β^0		
1. Cod 8/9 (+G)	30	18.1
2. IVS II.1 (G>A)	23	13.9
3. Codon 5 (-CT)	13	7.8
4. IVS I.1 (G>A)	12	7.2
5. Cod 8 (-AA)	11	6.6
6. Cod 44 (-C)	6	3.6
7. Cod 39 (C>T)	5	3.0
8. Cod 36/37 (-T)	2	1.2
9. Cod 25/26 (+T)	2	1.2
10. Cod 82-83 (-G)	1	0.6

In the current study, 34 different genotypes were determined; homozygous codon 8/9 (7.2%) was the most common, followed by homozygous IVS I-5, compound heterozygous IVS II-1/codon 8/9, and compound heterozygous IVS II-1/IVS I-5, all were in the same frequency (6.0%). Almost 80% of patients had inherited $\beta^0\beta^0$ or $\beta^0\beta^+$, while just 17 patients (20.5%) had inherited homozygous or compound heterozygous $\beta^+\beta^+$ genotypes (Table 3.19).

Table 3.19: Genotypes of 83 TM patients in the current study.

Genotypes	Frequency	Percent
β^0/β^0		
1. Cod 8/9 / cod 8/9	6	7.2
2. IVS II.1/ Cod 8/9	5	6.0
3. IVS I.1 / IVS I.1	4	4.8
4. Cod 5 / Cod 5	4	4.8
5. IVS II.1 / IVS II.1	3	3.6
6. Cod 8 / Cod 8	3	3.6
7. Cod 44 / Cod 44	3	3.6
8. IVS I.1 / Cod 8/9	3	3.6
9. Cod 39 / Cod 39	2	2.4
10. Cod 5 / Cod 8/9	1	1.2
11. Cod 8 / Cod 8/9	1	1.2
12. Cod 8 / IVS II.1	1	1.2
13. IVS I.1 / IVS II.1	1	1.2
14. Cod 5 / Cod 82-83	1	1.2
15. Cod 36/37 / Cod 36/37	1	1.2
β^+/β^+		
1. IVS I.5 / IVS I.5	5	6.0
2. IVS I.5 / IVS I.6	4	4.8
3. IVS II.745 / IVS II.745	3	3.6
4. IVS I.6 / IVS I.6	2	2.4
5. IVS I.110 / IVS I.110	2	2.4
6. IVS I.110 / IVS I.128	1	1.2
β^0/β^+		
1. IVS II.1 / IVS I.5	5	6.0
2. Cod 8/9 / IVS I.5	3	3.6
3. Cod 8/9 / IVS I.110	3	3.6
4. IVS II.1 / IVS I.110	3	3.6
5. IVS II.1 / IVS I.6	2	2.4
6. Cod 5 / IVS I.6	2	2.4
7. Cod 8 / IVS I.5	2	2.4
8. Cod 25/26 / IVS I.5	2	2.4
9. Cod 8 / IVS I.110	1	1.2
10. Cod 39 / IVS I.6	1	1.2
11. Cod 5 / IVS I.110	1	1.2
12. Cod 8/9 / -30	1	1.2
13. Cod 8/9 / IVS I.128	1	1.2

3.4 Disease Characteristics and Morbidities in TM and TI Patients

Patients with TM were diagnosed and transfused at an earlier age (0.8 ± 0.5 years), and (0.75 ± 0.5 years), respectively in comparison to TI patients (7.3 ± 6.9 years), and (6.1 ± 6.6 years), respectively *p*- value <0.001. Further, the vast majority of TM patients enrolled in this study received regular blood transfusions and iron chelation therapy, 97.6%, and 95.2%, respectively, while in contrast, 32.1% of TI patients required regular transfusions, and in fact, 20.7% had not been transfused (Figure 3.3). On the other hand, less than half of TI patients required occasional blood transfusions, though they had received hydroxyurea therapy. Additionally, of 83 patients who underwent splenectomy, 62.7% were TI patients. Furthermore, S. ferritin level ≥ 1000 µg/l was more frequently detected in TM patients, *p*-value <0.001 (Table 3.20).

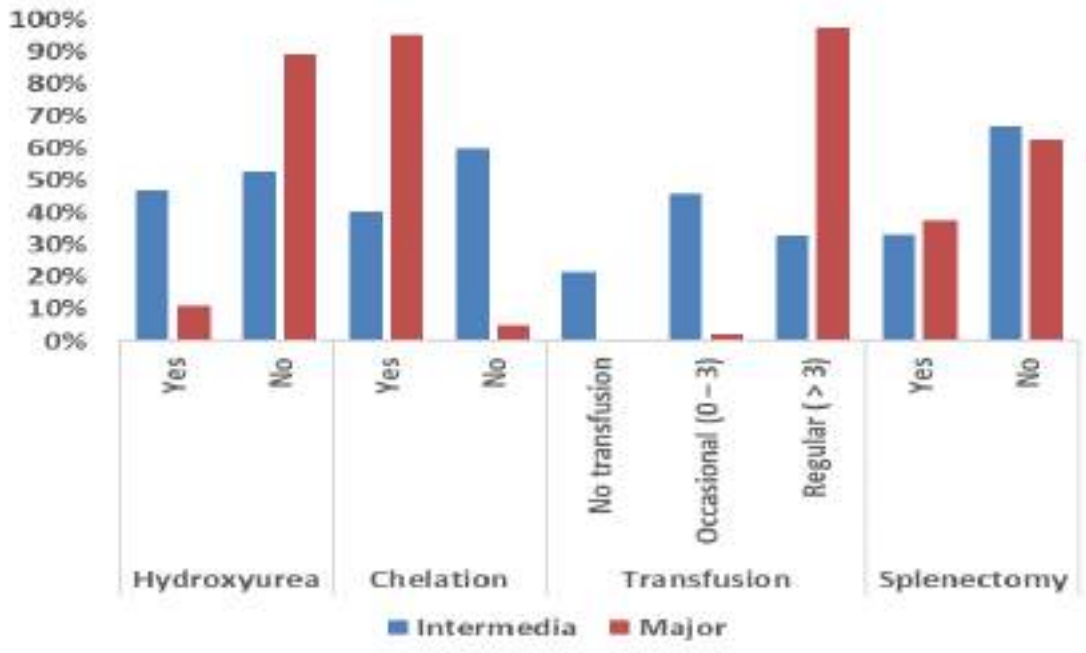

Figure 3.3: Clinical management of 242 thalassemia patients.

Table 3.20: Demographic and disease characteristics of 159 β-TI and 83 TM patients.

Parameter	TI (159)	TM (83)	P-value
Age (years)			
• <18	90 (56.6)	54 (65.1)	
• 18-34	54 (34.0)	28 (33.7)	<0.05
• ≥35	15 (9.4)	1 (1.2)	
Splenomegaly	93 (58.5)	30 (36.1)	<0.001
Mean spleen size ± SD	14.4 ± 3.4	12.5 ± 3.3	<0.001
Splenectomized	52 (32.7)	31 (37.3)	0.48
Hepatomegaly	60 (37.7)	27 (32.5)	0.26
Mean liver size ± SD	13.9 ± 2.5	13.5 ± 2.5	0.26
Serum ferritin (µg/dL)			
• <1000	122 (76.7)	26 (31.3)	<0.001
• ≥1000	37 (23.3)	57 (68.7)	
Treatment			
• None transfused	33 (20.7)	0 (0)	
• Occasional transfusion	75 (47.2)	2 (2.4)	<0.001
• Regular transfusion	51 (32.1)	81 (97.6)	
• Iron chelation	63 (39.6)	79 (95.2)	<0.001
• Hydroxyurea	75 (47.2)	9 (10.8)	<0.001
Complications			
• Bone disease	99 (62.3)	63 (75.9)	0.03
▲ Facial deformity	99 (62.3)	63 (75.9)	0.03
▲ Osteoporosis	17 (28.3)	20 (50)	0.02
• Endocrinopathies	44 (27.7)	34 (41)	0.08
▲ Growth retardation	25 (27.8)	(24 (44.4)	0.12
▲ Hypothyroidism	22 (16.8)	11 (13.4)	0.42
▲ Diabetes mellitus	(0)	5 (6.0)	0.002
• Hepatobiliary disease	46 (28.9)	24 (28.9)	0.99
▲ High ALT ≥50 IU/l	12 (7.5)	19 (23.2)	0.001
▲ Biliary complications	41 (25.8)	7 (8.5)	0.04
• Pulmonary hypertension	18 (11.3)	6 (7.2)	0.32
• Thrombosis	2 (1.3)	0 (0)	0.31
• EMH	1 (0.6)	0 (0)	0.47
• Leg ulcer	1 (0.6)	0 (0)	0.47
Rate of complications	122 (76.7)	69 (83.1)	0.25

Osteoporosis and hypothyroidism were evaluated in patients ≥10 years old and/or symptomatic [10], and growth retardation (height >2 SD below 3rd percentile for the mean age and gender) was evaluated in patients ≤18 years [188, 189].

Regarding disease-related morbidities, TM patients reported a significantly higher frequency of bone complications (both facial deformity and osteoporosis) as opposed to TI (Table 3.20), and (Figure 3.4). Likewise, abnormal LFT and growth retardation were more frequent in patients with TM and DM was only detected in this phenotype. In contrast, biliary complications, PHT, and hypothyroidism were more prevalent among TI patients, with thrombosis, EMH, and leg ulcers only encountered in this group.

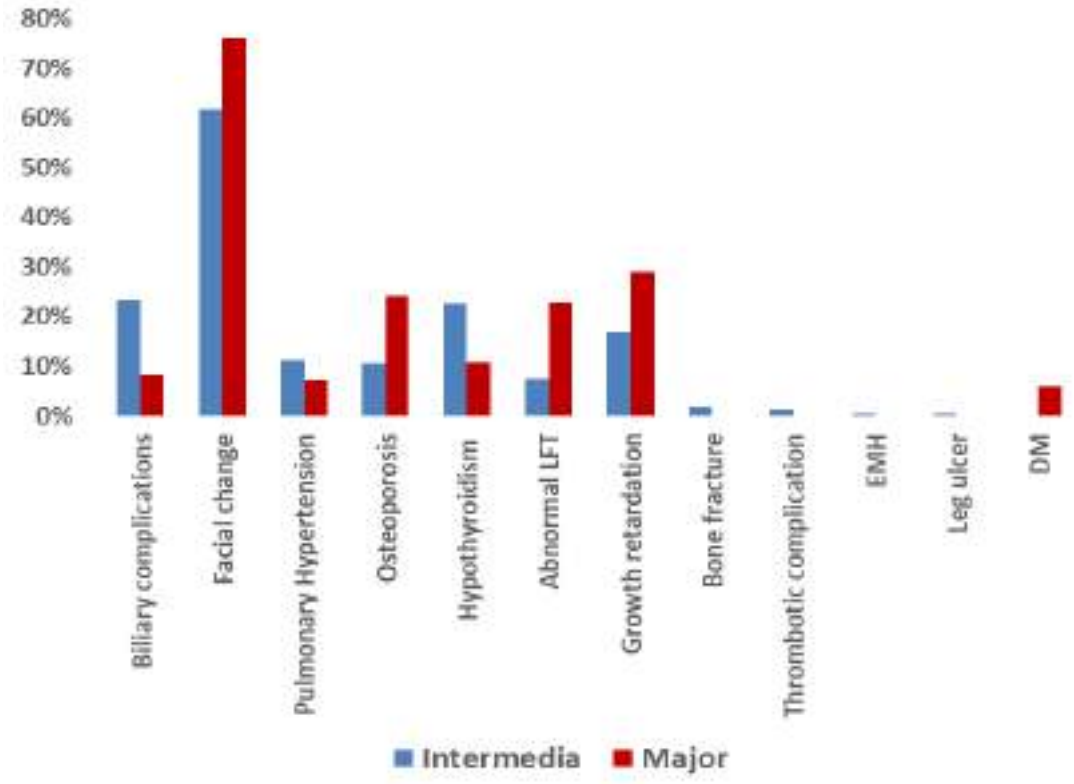

Figure 3.4: Distribution of disease-related morbidities among 242 β-thalassemia patients.

The reported complications rate in this study was 78.9%, with a slightly higher rate in TM in comparison to TI patients, 83.1% vs 76.7%, respectively (Table 3.20). Moreover, the rate of developing the disease-related morbidities increased with age, and this was particularly evident in TM patients (Figure 3.5).

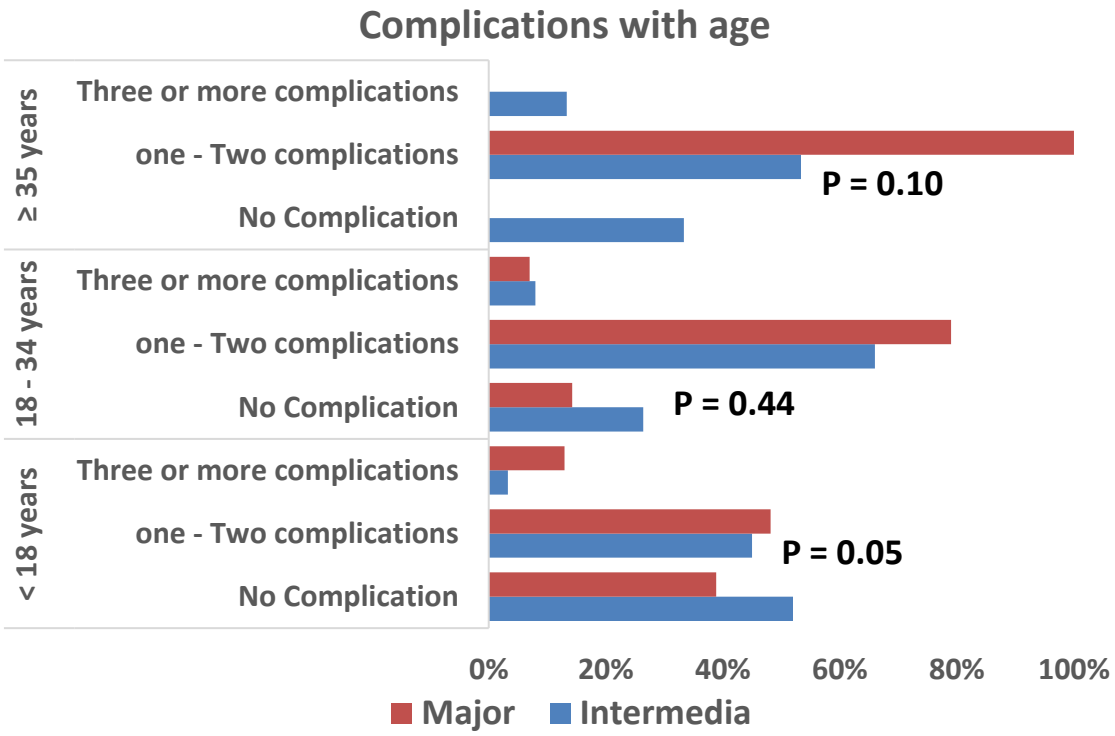

Figure 3.5: Frequency of disease-related morbidities at different age intervals.

CHAPTER FOUR

DISCUSSION

4.1 The Molecular Characterization of β-Thalassemia Mutations

The current study had evaluated the largest cohort of 242 β-thalassemia patients in Iraq and Kurdistan, composed of 159 β-TI and 83 β-TM from 162 families to investigate the molecular defect in β-globin gene and to explain the variable clinical course, proportion of disease complications and management options in both disease phenotypes.

The relative frequency and distribution of different mutation vary in different geographical locations, and the spectrum of β-thalassemia mutations that we determined in the current study were relatively wide, including those of Mediterranean, Asian-Indian, Kurdish, Turkish, Egyptian, and Saudi Arabian mutations. The four most frequent β-thal mutations identified were: IVS II-1 (G>A), followed by IVS I-6 (T>C), codon 8/9 (+G), and IVS I-110 (G>A) (35.7%, 18.0%, 8.5%, and 6.0% respectively).

IVS II-1 (G>A), a Mediterranean β^0-thal mutation was the most prevalent mutation detected in this study with the highest frequency (47.2%) among TI patients, in agreement with results that from central-Iraq Baghdad (41.2%) [200], as well as with different studies from Iran [201-203], including Iranian Kurds [204]. In contrast, previous studies from other parts of Kurdistan, Iraq revealed that IVS I-6 (a Mediterranean β^+ thal mutation) as the most frequent β-thal mutation among their β-TI patients, detected at around 33% [205, 206], which probably explains their higher reported

frequency of $\beta^+\beta^+$ or $\beta^0\beta^+$ genotypes. In addition, IVS I-6 was the most frequent β-thal mutation among TI patients in studies from Turkey [207], Lebanon [185], Egypt [208], Cyprus [209], and Italy [210], while it was the second common β-thal mutation reported by this study reported at (23.3%) of TI patients, in consistence with that of central-Iraq Baghdad study (24.0%) [200].

The third most recurrent mutation, codon 8/9 (+G) β^0 thal mutation was more frequent in TM patients (18.1%), followed by IVS I-5 (G>C) (15.7%), both are an Asian Indian β-thal mutation. The sequence of these 2 mutations were in accordance with an earlier study performed on 100 TM patients in 3 centers in Iraq; 2 centers in Baghdad (including Arab patients from middle and south of Iraq), and one center in Sulaymaniyah (Kurdish patients) [211], where codon 8/9 (39.5%) and IVS I-5 (26.3%) were the two most common reported mutation, with codon 8/9 was more frequent in the middle and north of Iraq (Sulaymaniyah), while IVS I-5 more occurred in the middle and south of Iraq (Basra). Furthermore, the frequency of codon 8/9 (+G) was in agreement with Iranian Kurdish population and northwestern Iran (15.7% and 14.5%, respectively) [204, 212], as well as an earlier study from Indian subcontinent [213]. However, IVS II-1 shown to be the most common β-thal mutation in TM patients in earlier studies from Iraq [214-216], Iran [202], and Kuwait [217], while it was the third common mutation among our TM patients (Table 3.10).

Further, IVS I-110 (a Mediterranean β^+ thal mutation) was the 4th most common mutation with relatively equal frequency between TI and TM phenotypes in our study, while it was the most common mutation in studies from Turkey [218], Egypt [219], and Lebanon [220]. The diversity in the relative

frequency and distribution of different mutations is more likely to be related to the multi ethnicity of the Iraqi population, geographical factor, genetic admixture, variable migration and interactions with the surrounding communities.

Despite that β^0 ($\beta^0\beta^0$) thal mutation rate had contributed to (56%) of β-TI genotypes in comparison to (47%) in TM genotypes, the former group of β-thal had an evidently less sever phenotype regarding the age of diagnosis, frequency of transfusion and the frequency of most of disease-related complications, which highly propose the coinheritance of disease modifiers such as single nucleotide polymorphism in the three major quantitative trait loci (QTLs) to induce Hb F synthesis and/or inheritance of α-thalassemia to modify the unbalance between α:β globin chains and subsequently ineffective erythropoiesis [221]. This has been particularly emphasized in studies from Iran, where β^0 are more frequent in β-TI patients and *Xmn*I polymorphism was found to be a considerable ameliorating factor [201, 202]. The frequency of β^+ thal mutation among β-TI patients in the current study was lower than figures from other parts of Kurdistan, Iraq (Duhok, 54.9%; Erbil, 60.2%) [205, 206], while it was approaching figures reported from central Iraq-Baghdad (49%) [200], also comparable to some extent to studies from India and Iran, where the inheritance of β^+ alleles was not responsible for the majority of the milder β-thal phenotypes [209].

Four new β-thal mutations were detected for the first time in Iraq, while reported by earlier studies from other parts of the world. The first is the frameshift codon (FSC) 25/26 (+T), a rare β^0-mutation, originally reported by Fattoum et al. at 1991 in a Tunisian family, where the insertion of thymidine between codons 25 and 26 in the first exon of β-globin gene had

resulted in a new termination codon (codon 26) and premature termination of the mRNA translation within the exon 1 of the β-globin gene, with associated minimal level of mutant mRNA in erythroid cells [(222)]. This mutation was co-inherited with IVS I-5 (β^+ mutation) in two patients with TM phenotypes.

The rest of 3 new mutations were among TI phenotype; the co-inheritance of single nucleotide polymorphism (SNP) +20 (C>T) β^+ mutation in the 5' untranslated regions (5' UTR) of the β-globin gene that is transcribed but not translated and is involved in posttranscriptional regulation of mRNA, co-inherited with IVS-II-745 (C>G), another β^+ mutation, in transposition rather than cis-position and resulted in thal intermedia phenotype in 2 siblings, otherwise co-inheritance of +20 in the cis position on the same allele would have resulted in a β-thal minor phenotype [(223)]. Additionally, these 2 siblings were investigated for the alpha gene triplication ($\alpha\alpha\alpha^{Anti3.7}$), both were negative for excess α-gene. In 2005, the HbVar database included the substitution C>T at nucleotide +20 in the promotor region 5' end flanking the first exon of the β-globin gene as a β^+ mutation, that produce a variable reduction of β-globin mRNA, in a heterozygous state, it would behave as a thalassemia trait, but when associated with IVS-II-745 mutation, it resulted in β-TI phenotype [(224)]. Both siblings were splenectomized, and first transfusion were at the age of 17 and 21 years, one of them is occasionally transfused while the other one had become transfusion dependent due to the alloimmunization.

The third new mutation was CAP +1 (A>C), a silent β^{++} Asian Indian mutation co-inherited with IVS-I-1 (β^0 mutation) resulting in a mild clinical phenotype in 2 siblings, they were just transfused once in their lifetime

during sever infection at the age of 3.5 and 10 years, respectively, both have mild splenomegaly, a mean Hb F was 67% and Hb A_2 2.2%.

Finally, a dominant-like β-thalassemia; Hb Housten resulted from a truncated protein due a nonsense mutation at codon 127 (A>G, Glutamine→ Proline) in exon III of the β-globin gene, resulting in hyper unstable Hb and thal intermedia phenotype in the heterozygous state, where the half-life of the abnormal Hb is limited to a few minutes or hours, thus rapidly after being synthesized, all the unstable Hb precipitates on the membrane of the red blood cell precursors in the bone marrow, resulting in an ineffective erythropoiesis. This observation shows the important role played by helix H in Hb stability, where its partial absence, or a large structural change, seems to be the major reason for the hyper instability of Hb [(225)]. This mutation was originally reported in an English family at 1991 [(226)]. Our patient was on occasional transfusion regimen (2-3 times/year), initiated at the age of 8 years and splenectomized at 33 years old, later became transfusion independent after splenectomy.

Among 242 β-thal patients, only 3 β-TI patients (2.0%) had inherited a single β-thal mutation, and this is consistent with previous studies worldwide where the large majority of cases of TI were homozygous or compound heterozygous to β-thal mutations [(187)]; namely, codon 127/wt (one patient), and IVS II-1/wt $\alpha\alpha\alpha^{anti3.7}$ in 2 patients. The later genotype had resulted in increased α:β imbalance, hemolysis, and ineffective erythropoiesis [(3)], and it has been implicated in TI in several earlier studies from Asia and the Mediterranean region [(187, 209, 210)].

4.2 Management Practice

4.2.1 Management of Thalassemia Major

Conventional management of TM patients at our Sulaymaniyah center is in compliance with clinical management guidelines of Thalassemia International Federation [(12)], which involves lifelong regular blood transfusions, usually administered every two to five weeks to maintain the pre-transfusion Hb above 9-10.5 g/dL. This transfusion regimen promotes normal growth, allows normal physical activities, adequately suppresses bone marrow activity in most patients, and minimizes transfusional iron accumulation [(156, 227)].

In addition, iron chelation therapy is administered to prevent iron related toxicity, with serum ferritin thresholds are commonly used to indicate the need for initiation or modification of iron chelation therapy, and maintaining ferritin concentrations lower than 1000 μg/L is most commonly used to indicate the need for initiation of iron chelation therapy [(12, 15, 142)]. Among 95.2% TM patients using iron chelation therapy, 68.7% of them had serum ferritin level ≥1000 μg/dL, with a mean ferritin level of (2006 ± 1665 μg/dL). Our figure was much lower than TM patients in northern Iraq (3822 ± 2921 μg/dL) [(228)], Egypt (3386 ± 1969 μg/dL) [(229)], and Turkey (4297 ± 2122 μg/dL) [(230)], while it was comparable to that of Iran (1876 ± 1790 μg/dL) [(231)]. This discrepancy most probably related to the degree of appropriate chelation therapy, type and compliance with different chelating drugs.

Likewise, our figure of splenectomy (37.3%) is within the range reported from Iraq and neighboring countries (11.3%-95%) [(23, 218, 231, 232)], though, the

indications of splenectomy needs to be revisited and restricted to selected cases to avert the risk of hypercoagulability, overwhelming infection post-splenectomy, and other serious disease-related morbidities [110].

4.2.2 Management of Thalassemia Intermedia

The transfusion regimen implemented in β-TI patients were individually tailored to meet patient's demands. When we compared our results with practices in TI management outlined by one of the first landmark studies "OPTIMAL CARE study" [90], which highlighted the management approaches in several Mediterranean and Middle Eastern countries (Table 4.1), we found that our patients were rather younger, less regularly transfused with lesser numbers of splenectomized and chelated patients. On the other hand, hydroxyurea therapy was prominently implemented at our center. Furthermore, our patients were more regularly transfused than other thalassemia centers in Iraq (Duhok and Basra) [206, 233], Lebanon [185], Iran [234], and Italy [105], with a much higher rate of splenectomy than recent reports from Sri Lanka (12%) [235], and Qatar (7%) [236].

Table 4.1: Comparison of some clinical parameters, treatment options, and disease-related complications between 159 β-TI patients in the current study and some other related studies.

Parameter	**Current Study (n=159)**	**Lebanon (n= 73) 2000**	**Optimal Care (n=584) 2010**	**Iran (n-153) 2011**	**Basra (n=80) 2013**	**Duhok Study (n=74) 2014**	**Italy (n=70) 2014**	**Sri Lanka (n=50) 2019**
Splenectomized	32.7	59	55.7	46.9	*	23.0	49	12.0
Serum Ferritin (µg/L)								
<1000 ≥1000	76.7 23.3	*	64.4 35.6	*	55 45	67.6 32.4	*	*
Treatment								
Never transfused Occasional T. Regular T.	20.8 47.2 32.1	28.8 58.9 12.3	23.8 24.5 51.7	27.5 45.5 27	21.2 78.8	32.4 51.4 16.2	53 34 13	4.0 42 44
Iron chelation	39.6	*	47.5	*	*	14.9	56	46
Hydroxyurea	47.2	*	34.6	*	*	2.7	16	*
Complications								
Facial Deformity	62.3	44	*	*	*	73	*	*
Osteoporosis	28.3	*	22.9	53	30.0	*	49	*
Growth retardation (height <3rd percentile)	27.8	*	*	*	42.5	31.3	*	26.7
Subclinical Hypoth.	16.8	*	*	*	*	*	7.1	*
Cholelithiasis	13.8	*	17.1	9.8	2.5	*	*	10.0
PHT	11.3	*	11	23.5	5.0	20.4	*	33.3
Abnormal liver function	7.5	*	9.8	29.3	*	13.5	*	*
Bone Fracture	1.9	*	*	0.8	*	0	18	0.0
Thrombosis	1.3	*	14	2.9	2.5	0	*	0.0
References	Current study	(185)	(90)	(234)	(233)	(206)	(105)	(235)

* Not mentioned in the study.

Despite the lower frequency of chelation therapy used in β-TI patients in this study, over three fourth had mean ferritin value <1000 μg/L, a figure that is higher than the reported value of the "OPTIMAL CARE study" [(90)], and those from previous figures Iraq [(206, 233)] (Table 4.1). Such a finding could be possibly attributed to the use of hydroxyurea among a higher proportion of our β-TI patients (47.2%), which had improved the α:β chain imbalance and subsequently improved the ineffective hemopoiesis [(10)].

Interestingly, our data on hydroxyurea therapy in thalassemia patients is supporting previous encouraging results from "OPTIMAL CARE study" [(90)], and a single report evaluating 6 years of hydroxyurea therapy in TI patients in Iran [(237)]. Eighty-four β-thal patients (including 75 TI and 9 TM) used this Hb F inducer and revealed a potentially lower S. ferritin, annual transfusion frequency, and chelation therapy requirement, Table 3.13 further supporting the earlier proposed role of hydroxyurea in improving α: β globin chain imbalance and eventually more effective erythropoiesis.

4.3 Disease-related Complications

β-thal patients had many clinical complications reported in this study. The pathophysiology is multifactorial due to the interaction of ineffective erythropoiesis, iron overload, and chronic tissue hypoxia (chronic hemolytic anemia) [(238)]. Disease complications were encountered in 78.9% of the enrolled patients and the rate was more frequent among TM patients. The discrepancy in the rates of multimorbidity profile of TM and TI reported in various studies (Table 4.1) had been attributed to the difference in the underlying genotype and clinical management lines (i.e., transfusion frequency, chelation, more frequent splenectomy and the use of fetal

hemoglobin inducing therapy) in thalassemia centers in Iraq and worldwide [90, 238].

4.3.1 Bone Disease

Bone abnormalities (osteoporosis and facial deformity), was the most prevalent morbidity documented in this study (66.9%). Chronic anemia, enhanced ineffective erythropoiesis, and consequent bone marrow expansion were directly implicated in addition to splenectomy, as well as low fetal hemoglobin [105, 238]. The disease-related morbidity was detected at a significantly higher frequency among our enrolled TM patients, while it was reported to be more profound in β-TI in previous studies [89, 90, 231]. This variation might be attributed to the more frequent use of hydroxyurea in just less than half of our TI patients, with further support by iron chelation therapy (39.6% of TI patients) to lower the rate of bone complications to 62.3% vs 75.9% in TM, *p*-value = 0.03, in consistence with previous studies [90, 238]. Despite regular long-term transfusions and iron chelation therapy, particularly in TM patients, yet thalassemic patients continue to lose bone mineral density (BMD) over time, suggesting that underlying genetic factors play a significant role in the imbalance of bone remodeling [239]. This high prevalence of bone complications warrants close follow-up with annual assessment of BMD by DEXA scan as recommended by TIF [10], with early and appropriate initiation of therapy.

4.3.2 Endocrinopathies

Endocrine abnormalities are among the most common complications of β-thalassemia [240], and was the second common complication (32.2%) among our β-thal patients. The lower prevalence of endocrine diseases in TI patients 27.7% vs. 41% in TM patients (Table 3.12), might be related to the lower extent of blood transfusion, slower iron accumulation rate and hepatic predominance of iron-loading [105], in agreement with our figures (Figure 3.3).

Moreover, growth retardation figure in TI patients (27.8%) was lower in comparison to previous reports from Iraq [206, 233], while it was comparable to that of Sri Lanka [235] (Table 4.1). This might be attributed to younger age and a lower proportion of splenectomized TI patients in this study as intact spleen might be a reservoir of excess body iron in addition to its scavenging effect on iron-free fraction, including non-transferrin bound iron [241]. Regarding TM, the prevalence of growth retardation was lower than Egyptian study (68.5%) [229], while in accordance with that of TM in Turkey (40.6%) [230].

Unlike previous reports [90, 106], hypothyroidism (97.1% subclinical) was diagnosed at a relatively higher frequency in (16.8%) TI compared to (13.4%) TM patients (Figure 3.3). The incidence of subclinical hypothyroidism among our β-TI patients, an iron-overload-related morbidity, was in accordance with previous studies from Egypt (16.7%) [242], and from Iran (19%) [243], while it was higher than an Italian figure of 7.1% [105] (Table 4.1). On the other hand, the incidence of hypothyroidism

among our TM patients was higher than Turkey (5.2%) [230], and Egypt (9.6%) [229].

As for DM, it was also observed in well transfused and regularly chelated TM patients [12], suggesting the role of other factors in the pathogenesis including individual sensitivity to iron and chronic anemia. Five (2.1%) patients were identified with DM in this study, just among TM; two had S. ferritin ≥1000 μg/l. This figure was in accordance with previous reported studies from Iraq (2.3%) [23], and Turkey (2.5%) [230], while much lower than Egypt (15.1%) [229].

4.3.3 Hepatobiliary Complications

Among different body organs susceptible to damage in β-thalassemia patients, the liver represents a major target and iron overload is considered as the most important single cause, while HCV infection is the second acting in synergy, particularly to increase the risk of hepatocellular carcinoma. Drug-toxicity (chelation therapy) is an added risk factor [84, 85].

The total frequency of hepatobiliary complications [abnormal liver function test (LFT); alanine transaminase (ALT ≥50 IU/l) and biliary complications; cholelithiasis and cholecystectomy] in this study was the same for TM and TI patients, at 28.9% (Table 3.12). However, abnormal LFT was significantly prevalent among TM (23.2%) in comparison to (7.5%) TI patients, *p* value <0.05, which can explained by a significantly higher frequency of raised S. ferritin ≥1000 μg/l, HCV infection, together with regular iron chelation therapy requirement in TM (68.7%, 26.8%, and 95.2%, respectively), in comparison to (23.3%, 11.3%, and 39.6% respectively) in TI patients (Table 3.12), and supported by previous studies

[85, 244]. The higher frequency of biliary complications observed in TI had been supported by earlier studies [232, 238], and is attributed to the underlying chronic hemolytic anemia [19]. Furthermore, it was significantly reported among splenectomized patients, and patients aged ≥18 years (Table 3.3), in agreement with earlier results [19, 90, 245]. Furthermore, 75 (31%) β-thal patients enrolled had hepatomegaly with normal LFT, half of them used iron chelation therapy, 25.3% were positive for HCV infection and 22.7% had S. ferritin ≥1000 µg/l (Table 3.16). In view of the above findings, a monthly follow-up of hepatic transaminases is justified for the early detection of hepatic complications.

4.3.4 Pulmonary Hypertension and Thrombosis

Another potential complication reported in this study was PHT, a disease progression complication in the absence or with an improper blood transfusion. Pulmonary hypertension was reported at higher frequency in TI patients than TM in consistence with other studies [101, 232], though the rate (11.3%) was much lower than Duhok (northern Iraq) (20.4%) [206], Iran (23.5%) [234], and Sri Lanka (33.3%) rates [235], it was in consistence with "OPTIMAL CARE study" (11.0%) [90] (Table 4.1). Likewise the frequency of PHT among our TM patients (7.2%) was much lower than figure reported from Erbil (31%) [228], Egypt (40%) [246], and Iran (47.2%) [247], while it was in agreement with Italian figure (10%) [248]. The discrepancies in PHT prevalence is attributed to variation in diagnostic techniques used to measure the pulmonary arterial pressure, differences in sample size and characteristics of studied population [248].

Splenectomy in thalassemia had been considered a significant risk factor for many disease-related complications, in particular, thrombosis and PHT [108]. The development of these complications has been contributed to the presence of high platelet counts and aggregation after splenectomy [249] and/or to increased number of RBCs with negatively charged membranes that carry thrombogenic potential [250]. In addition, in splenectomized TI patients, thrombin generation is significantly higher than in control subjects and patients who had not undergone splenectomy [109]. In our group, two patients (0.8%) had documented thrombosis, just detected in β-TI and both were splenectomized. The low incidence of thrombosis may be explained by younger age and the possibility of non-documented asymptomatic cases of thrombosis. Furthermore, chronic thromboembolism in splenectomized thalassemia patients was linked with a high frequency of PHT [81, 251], in accordance with our result (Table 3.14). In the light of the above morbidities associated with splenectomy and despite the advantage of splenectomy in maintaining higher Hb levels, clinical practice is gradually shifting to restrict splenectomy indications into; growth retardation, hypersplenism with symptomatic leukopenia, and/or thrombocytopenia or symptomatic hypersplenism [43, 238]. Besides, this study had revealed that the rate of PHT was frequently observed in patients used chelation therapy in contrast to other reports [90, 101], where iron chelation had reduced the incidence of PHT. This finding can be attributed to the small sample size of patients (24) with PHT, where (19) 80% of them using chelation therapy. No patient with heart failure is detected in the current study.

4.3.5 Other Complications

Documented radiological evidence of extramedullary hemopoiesis (EMH) was detected in just one (0.6%) β-TI patient. The extramedullary hematopoietic masses occur almost exclusively in TI patients compared to TM (particularly when transfusion is inadequate), 20% vs. <1% [90, 115]. Our figure was lower than Duhok (northern Iraq) (2.7) [206], and much lower than "OPTIMAL CARE study" (21.2%) [90]. Furthermore, chronic leg ulceration was also detected in one (0.6%) β-TI patient, in accordance with our study, TI patients have higher risk of developing leg ulcers, particularly in poorly controlled disease, as compared to the regularly transfused TM patients [112].

Despite compliance with the management guidelines for thalassemia patients, we have reported a high complication rate, with an increased probability of complications with advanced age (Figure 3.2), a result which had been suggested by a few previous studies [90, 238]. Such findings justify the initiation of Sulaymaniyah Premarital Screening Program in 2006 coupled with genetic counselling and followed by anti-natal screen few years later to screen at risk couples and prevent the birth of affected fetus.

4.4 Limitations of The Study

1. The current study lacked the estimation of different genetic modifiers including concomitant α-thalassemia and polymorphism at QTL due to a limited financial budget.
2. Using serum ferritin to estimate the iron overload instead of liver iron concentration by T2 or R2 magnetic resonance imaging, which may underestimate the actual iron burden [(87)]. Serum ferritin assessment is widely available and might be the only assessment that is affordable in resource-poor countries, including Iraq.
3. Measuring pulmonary artery systolic pressure (PASP) by Doppler Echocardiography to determine PHT instead of right heart catheterization, which may increase the rate of false-positive findings. However, echocardiography is still the modality of choice used in many studies on thalassemia for financial/practical reasons and relying on reports of good relationship between Doppler estimates and invasive measurement of PASP [(96, 97, 252)].
4. This study included a limited number of TM patients in comparison to TI despite the predominance of the abovementioned cases at our local thalassemia center. This might be again attributed to financial issues and to the fact that we aimed to shed more light on TI patients as such patients were not addressed earlier.

4.5 Conclusions:

1. The current study, the largest from Iraq and Kurdistan region on β-thalassemia patients, revealed that β^0 was the most frequent β-thal mutations, a result that is rather distinct from reports from other parts of Iraq and nearby countries.
2. Homozygous mutations were determined in (76.3%) patients, 62.9% of which were the result of consanguinity.
3. We detected a notable difference in the type and relative frequency of different mutations in TI and TM patients. The most frequent β-TI mutation was IVS II-1 (G>A), followed by IVS I-6 (T>C), while the most frequent TM mutation was codon 8/9 (+G), followed by IVS I-5 (G>C).
4. Four new β-thal mutations were detected for the first time in Iraq, namely; codon 25/26 (+T) in TM, with 3 new mutations in TI patients, including coinheritance of +20 (C>T) with IVS-II-745 (C>G), CAP +1 (A>C), and a dominant-like β-thalassemia; Hb Housten (codon 127 A>G).
5. Interestingly, using hydroxyurea therapy (Hb F inducing drug) among our β-TI patients implemented at Sulaymaniyah Thalassemia Center resulted in a potentially lower S. ferritin, a lower annual transfusion frequency, and a less chelation therapy requirement in comparison to previous studies from other parts of Iraq, surrounding, and other Mediterranean countries.
6. Disease-related complications were encountered in 78.9% of the enrolled β-thalassemia patients despite adherence to TIF regulations. Additionally, the rate was more frequent among TM patients, with an

evidently higher rates in patients with $\beta^0\beta^0$, and $\beta^0\beta^+$ genotypes, with increased probability of developing complications with advanced age.

7. Bone abnormalities were the most prevalent morbidity documented in this study, followed by endocrinopathies, hepatobiliary complications, and PHT, opposed to DM, thrombosis, EMH and leg ulcer that were the least prevalent morbidities.
8. In multivariate analysis among 159 β-TI patients, iron chelation therapy was protective for a multitude of disease-related complications, while hydroxyurea therapy, transfusion, and mean serum ferritin ≥1000 μg/L were independently associated with an increased risk for osteoporosis.

4.6 Recommendations:

1. The role of genetic modifiers of disease severity in TI patient needs to be addressed in future studies for a better understanding of the underlying pathophysiology.
2. A significant proportion of our β-thalassemia patients developed disease-related complications, which necessitates serious closer follow up with earlier and a timely effective medical interference to curtail these complications before reaching the point of irreversibility.
3. Regular monitoring of iron burden by direct non-invasive estimation of liver iron concentration by MRI, considering the strong correlation with total body iron stores.
4. Since access to blood for transfusion therapy in limited-resource countries is a challenge and poses a considerable health burden, a continuous financial support to regional preventive programs is the key to reduce the number of affected births in thalassemia high prevalence settings.

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Appendices

Publication

1. Shaema Salih Amin, Sana Dlawar Jalal, Kosar Muhammed Ali, Ali Ibrahim Mohammed, Luqman Khalid Rasool, and Tara Jamel Osman. Beta-Thalassemia Intermedia: A single Thalassemia Center Experience from Northeastern Iraq. It has been published in journal (BioMed Research International), listed in (Thombson Reuters-Clarivates Analytics), IF=2.2, volume 2020, Article ID 2807120, 11 pages.
2. Shaema Salih Amin, Sana Dlawar Jalal, Kosar Muhammed Ali, Luqman Khalid Rasool, Tara Jamel Osman, Omed Hameed Ali, and Abdalhamid Saber M-Saeed. Molecular and Disease-Related Morbidities of β-Thalassemia Patients from the Northeastern Part of Iraq. It has been accepted for publication in journal (International Journal of General Medicine), on 16. November.2020. IF=1.9. Article ID 277947.

Appendix A

Questionnaire Form:

Date of Interview:	
Name of the patient:	Patients cod number:
Date of birth:	Age:
Gender:	Mobile No.
Residence address:	Ethnicity:
Marital status:	
Consanguinity:	
Occupation:	
Age of first blood transfusion	
Age at diagnosis	
Frequency of transfusion from the beginning of the disease and in the last year	
Family history of thalassemia	
Number and codes of the affected siblings	
History of splenectomy	Age at splenectomy
History of transfusion after splenectomy	
History of cholecystectomy	Age at cholecystectomy
Height (cm):	
Weight (Kg):	

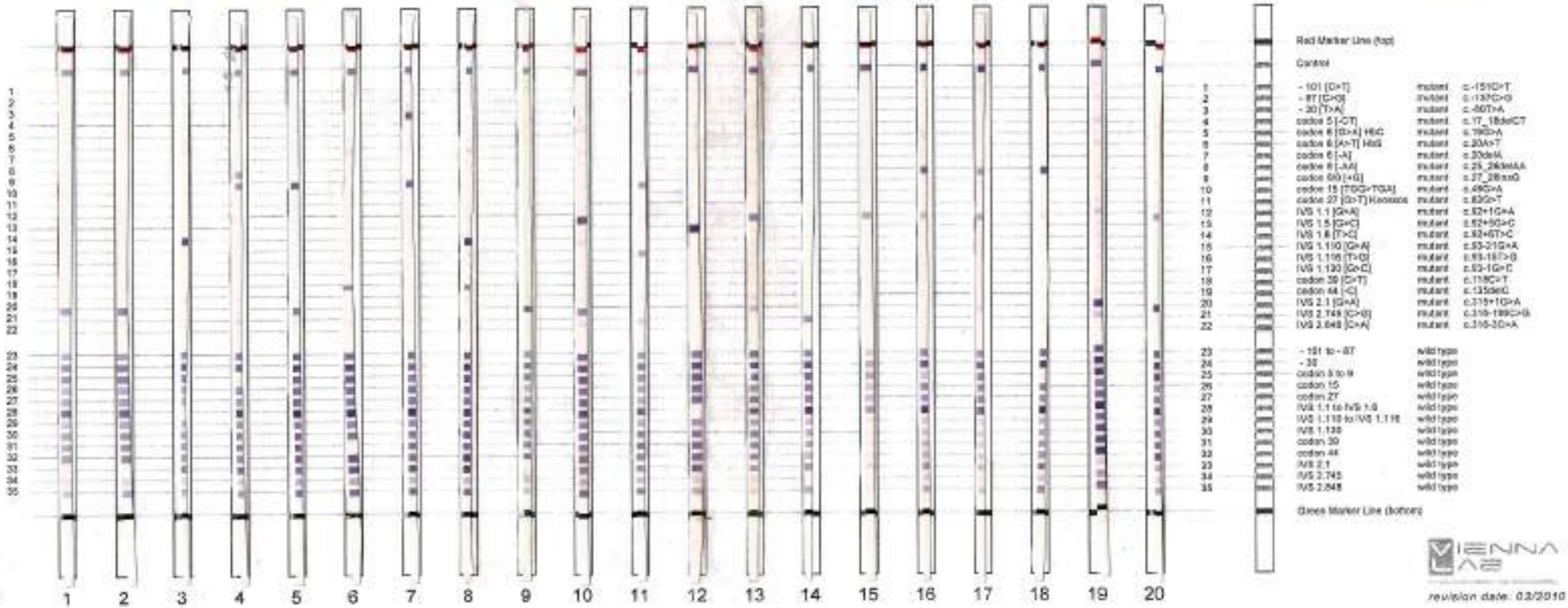
Thalassemic facial deformity
CBC: Hb (g/dL) PCV (fL) MCH (pg)
Hemoglobin electrophoresis: • Hb A (%) • Hb A_2 (%) • Hb F (%)
Liver function test: • ALT (IU/L) • AST (IU/L) • TSB (mg/dl) • S. alkaline phosphatase (IU/L)
Serum ferritin level (μg/L)
Virological investigations: • HBV • HCV • HIV
Blood sugar (g/dl) History of diabetes mellitus:
Thyroid function test: • TSH • Free T4
DEXA scan for evaluation of osteoporosis:
Echocardiography for PHT detection:
Documented EMH:
Documented venous thrombosis:
Ultrasound Findings: • Liver size • Spleen size • Gall bladder stone
Type and duration of chelation therapy in the last year:

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	163	20 + 33	homozygous IVS 2.1	10.7.2018
2	831	20 +33	homozygous IVS 2.1	10.7.2018
3	900	14 +28	homozygous IVS 1.6	17.7 2018
4	8962	8 +9 +25	cod 8/ cod 8/9 + cod 8/9	17.7.2018
5	30	9 + 20	cod 8/9 / IVS 2.1	17.7.18
6	781	18 +31	cod 44/44	17.7.18
7	830	3 + 9	-30 / cod 8/9	17.7.18
8	1173	14 +18	IVS 1.6 / cod 39	17.7.18
9	7219	20+33	IVS 2.1 / 2.1	17.7.18
10	9517	12 + 20	IVS 1.1 / IVS 2.1	17.7.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	183	9 + 15	cod 8/9 / IVS 1.110	31.7.18
12	384	13 + 28	homo IVS 1.5	31.7.2018
13	756	12 +20	homo IVS 2.1 / IVS 1.1	31.7.18
14	1124	21 + 34	homo IVS 2.745	31.7.2018
15	761	12 +	IVS 1.1 /	31.7.18
16	1352	8 +12	cod 8 / IVS 1.1	31.7.18
17	9315	8 +12	cod 8 / IVS 1.1	31.7.18
18	8767	8+25	homo cod 8	31.7.18
19	1328	20 + 33	IVS 2.1 / 2.1	7.8.18
20	9342	20 +12	IVS 2.1 / 1.1	7.8.18

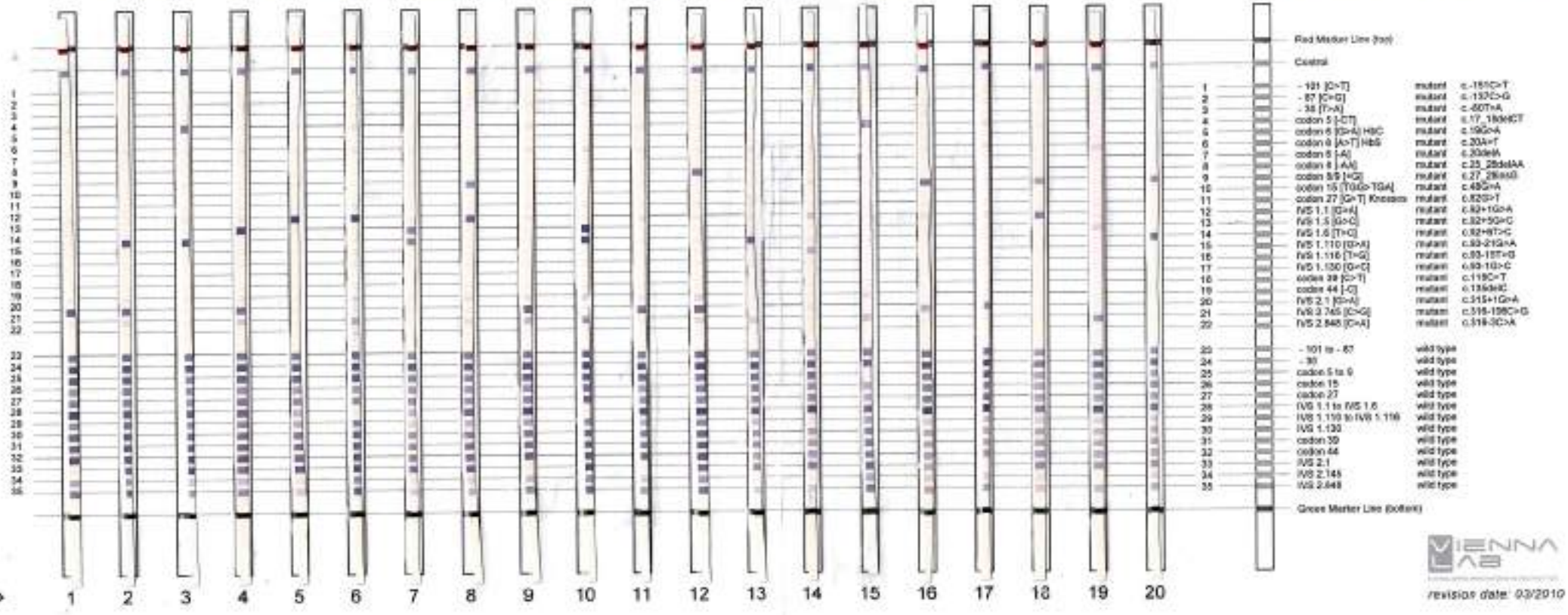


Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	17	20 + 33	IVS 2.1 / 2.1	7.8.18
2	1172	14 + 20	IVS 1.6 / 2.1	7.8.18
3	1099	4 + 14	cod5/IVS 1.6	7.8.18
4	1293	13 + 2	IVS 1.5 / 2.1	7.8.18
5	9364	12 +	IVS 1.1 /	7.8.18
6	417	12 + 28	IVS 1.1 / 1.1	7.8.18
7	809	13 + 14	IVS 1.5 / IVS 1.6	14.8.18
8	107	9 + 12	cod 8/9 / IVS 1.1	14.8.18
9	569	20 + 33	IVS 2.1 / 2.1	14.8.18
10	347	13 + 14 + 28	IVS 1.5 / IVS 1.6	14.8.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	1175	20 + 3	IVS 2.1 / 2.1	14.8.18
12	533	8 + 20	cod 8 / IVS 2.1	14.8.18
13	20086	14 + ?	IVS 1.6 / unclear	14.8.18
14	488	15 + 29	IVS 1.110 / 1.110	14.8.18
15	937	4 + 25	cod5 / cod5	30.8.18
16	5682	9 + 20	cod 8/9 / IVS 2.1	30.8.18
17	1979	20 + 33	IVS 2.1 / 2.1	30.8.18
18	39	9 + 12	cod 8/9 / IVS 1.1	30.8.18
19	269	21 + 34	IVS 2.745 / 2.745	30.8.18
20	446	9 + 14	cod 8/9 / IVS 1.6	30.8.18



VIENNA LAB

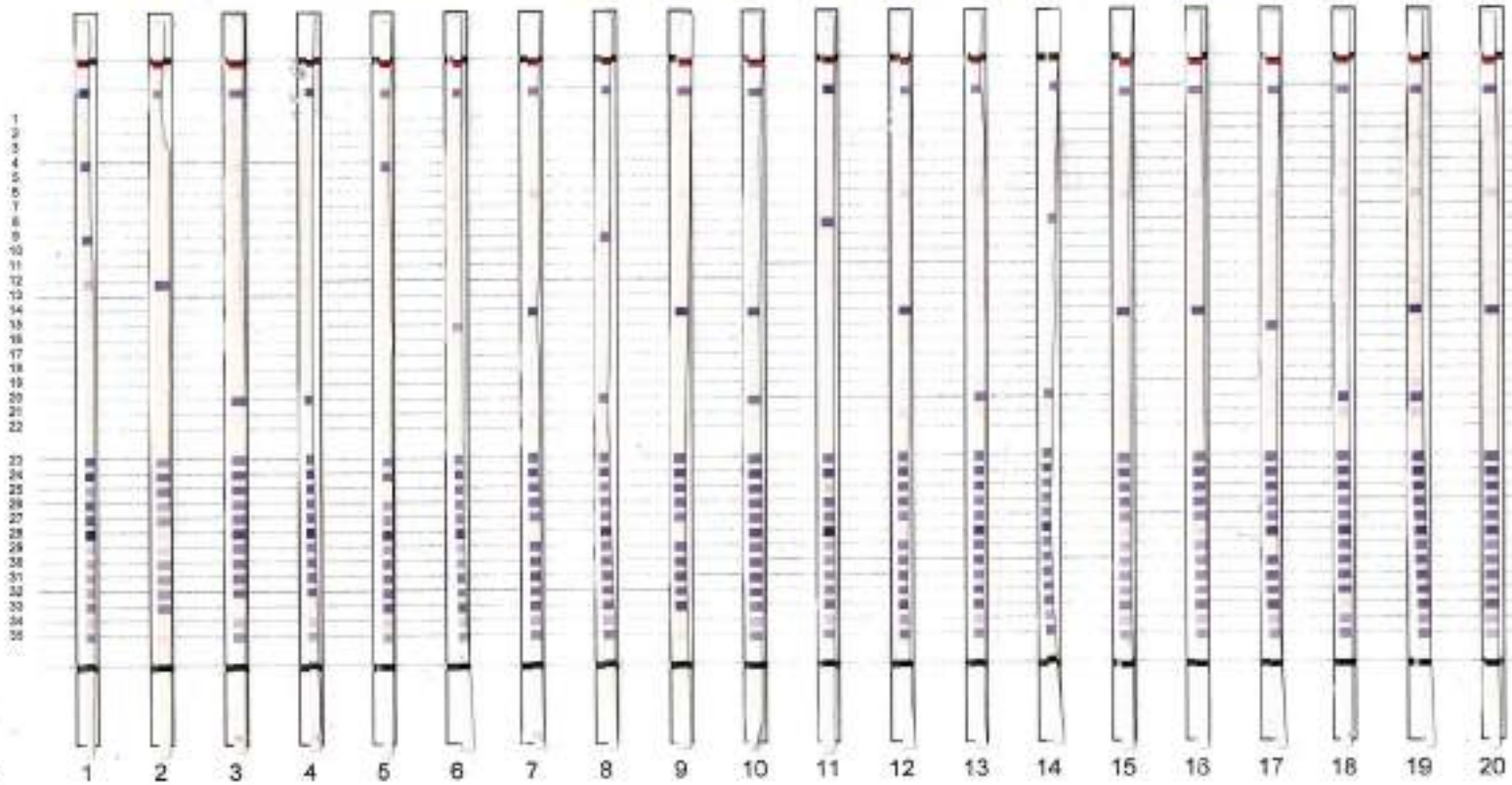
revision date: 03/2010

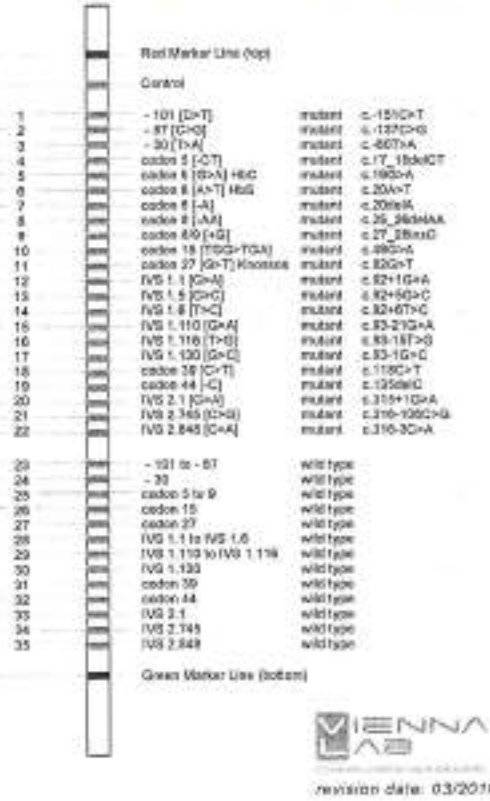
Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	450	4 + 9	cod5/c8/9	30.8.18
2	25	12 + 28	IVS 1.1/IVS 1.1	30.8.18
3	1228	20 + 33	IVS 2.1/2.1	18.9.18
4	878	20 + 33	IVS 2.1/2.1	18.9.18
5	427	4 + 25	cod5/cod5	18.9.18
6	786	15 +	IVS 1.110/	18.9.18
7	147	14 + 28	IVS 1.6/IVS 1.6	18.9.18
8	80	9 + 20	cod8/9/IVS2	18.9.18
9	8145	14 + 28	IVS 1.6/IVS 1.6	18.9.18
10	1276	14 + 20	IVS 1.6/IVS 2.1	18.9.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	913	8 + 25	cod8/cod8	18.9.18
12	890	14 + 28	IVS 1.6/IVS 1.6	9.10.18
13	376	20 +	IVS 2.1/	9.10.18
14	5183	8 + 20	cod8/IVS 21	9.10.18
15	977	14 + 28	IVS 1.6/IVS 1.6	9.10.18
16	855	14 + 28	IVS 1.6/IVS 1.6	9.10.18
17	522	15 + 29	IVS 1.110/IVS 1.110	9.10.18
18	9028	20 + 33	IVS 2.1/IVS2	9.10.18
19	1127	14 + 20	IVS 1.6/IVS 2.1	16.10.18
20	1326	14 +	IVS 1.6/	16.10.18





VIENNA LAB

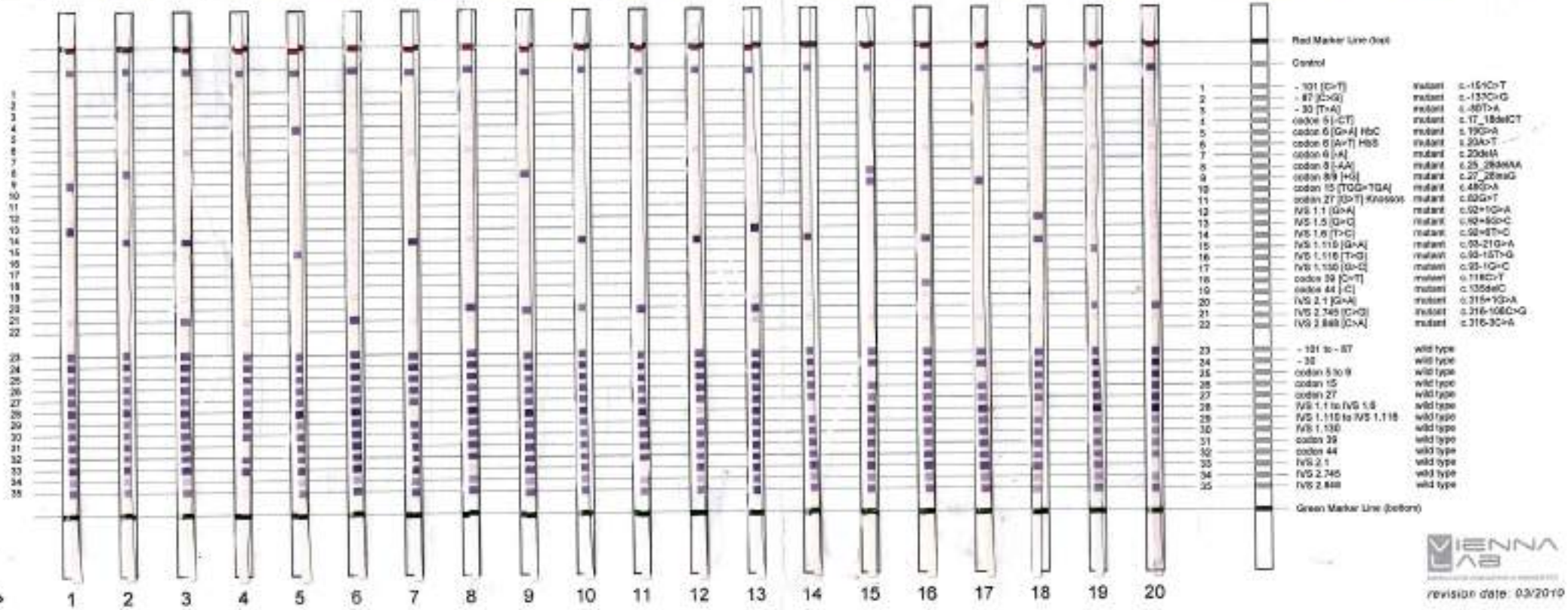
revision date: 03/2010

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	440	9 +13	cod 8/9 / IVS 1.5	16.10.18
2	9071	8 +14	cod 8 / IVS 1.6	16.10.18
3	1346	14 +21	IVS 1.6 / IVS 2.745	16.10.18
4	1321	31+	cod 36/37 / cod 36/37	16.10.18
5	1270	4 +15	cod 5 / IVS 1.110	16.10.18
6	695	21 +34	IVS 2.745 / 2.745	23.10.18
7	709	14 + 28	IVS 1.6 / IVS 1.6	23.10.18
8	286	20 +33	IVS 2.1 / IVS 2.1	23.10.18
9	136	8 +20	cod 8 / IVS 2.1	23.10.18
10	795	14+20	IVS 1.6 / IVS 2.1	23.10.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	176	20 +33	IVS 2.1 / IVS 2.1	23.10.18
12	985	14 + 28	IVS 1.6 / IVS 1.6	23.10.18
13	113	13 + 20	IVS 1.5 / IVS 2.1	23.10.18
14	1313	14 + 28	IVS 1.6 / IVS 1.6	30.10.18
15	984	8+9+25	cod 8 / cod 8/9	30.10.18
16	9361	14+18	IVS 1.6 / cod 39	30.10.18
17	309	9 +25	cod 8/9 / cod 8/9	30.10.18
18	954	12+14+28	IVS 1.1 / IVS 1.6	30.10.18
19	962	15+20	IVS 1.110 / IVS 2.1	30.10.18
20	9241	20+33	IVS 2.1 / IVS 2.1	30.10.18



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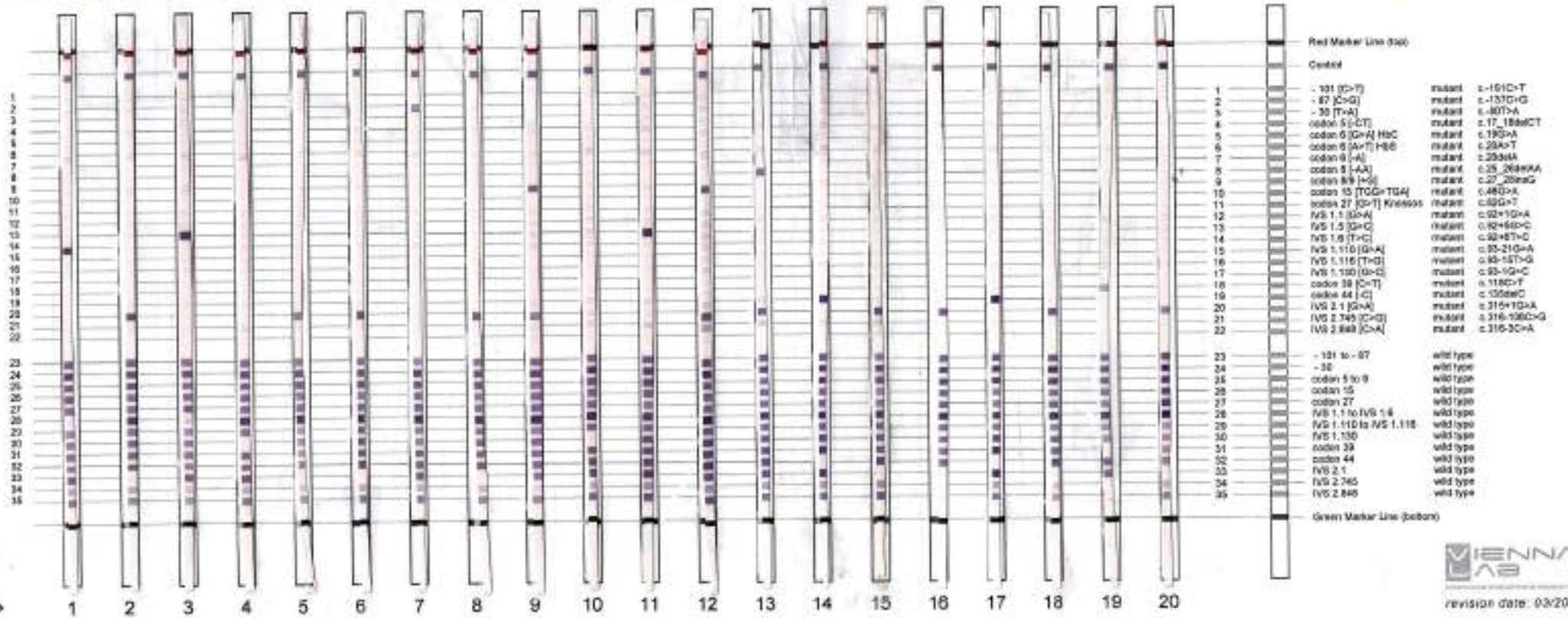
Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	895	14 +28	IVS 1.6/IVS 1.6	8.11.18
2	237	20 +33	IVS 2.1/IVS 2.1	8.11.18
3	798	13 +28	IVS 1.5/IVS 1.5	8.11.18
4	940	30 +	IVS 1.28	8.11.18
5	460	20 + 33	IVS 2.1/IVS 2.1	8.11.18
6	992	20 +33	IVS 2.1/IVS 2.1	8.11.18
7	661	2 +	-87/	8.11.18
8	928	20 +33	IVS 2.1/IVS 2.1	8.11.18
9	9068	9 +20	cod 8/9/IVS 2.1	8.11.18
10	1112	30 +	IVS 1.28/	8.11.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	576	13 +	IVS 1.5/	8.11.18
12	626	9 +20	cod 8/9/IVS 2.1	8.11.18
13	184	8 +20	cod 8/IVS 2.1	5.12.18
14	1164	19 +32	cod 44/cod 44	5.12.18
15	1350	20 +33	IVS 2.1/IVS 2.1	6.12.18
16	772	20 +33	IVS 2.1/IVS 2.1	6.12.18
17	546	19 +32	cod 44/cod 44	6.12.18
18	129	20 +33	IVS 2.1/IVS 2.1	6.12.18
19	422	18 +31	cod 39/cod 39	2.12.18
20	1220	20 +33	IVS 2.1/IVS 2.1	6.12.18



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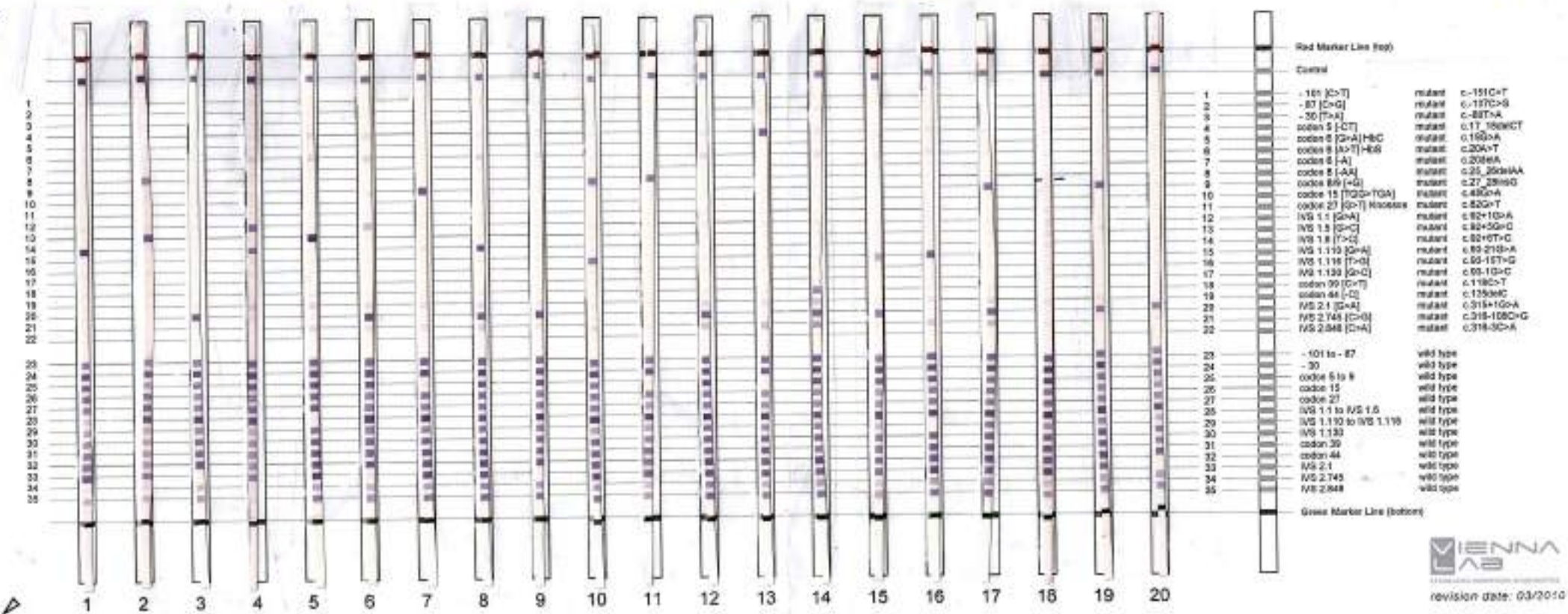
revision date: 03/2019

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	9514	14+28	IVS1-6/ IVS1-6	6.12.18
2	182	8 + 13	cod 8/IVS 1-5	6.12.18
3	17	20 + 33	IVS 2-1/ IVS 2-1	6.12.18
4	1034	12 +14	WS 1-1/ IVS 1-6	9.12.18
5	724	13+ 28	IVS 2-1/ IVS 2-1	9.12.18
6	6494	20 +33	IVS 2-1/IVS 2-1	9. 12. 18
7	1107	9 +25	cod 8/9/cod 8/9	9.12.18
8	9625	14 + 20	IVS 1-6/IVS 2-1	9.12.18
9	162	20 +33	IVS 2-1/ IVS 2-1	9.12.18
10	775	8 +15	cod 8/IVS 1-110	9.12.18

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	7131	8 + 25	cod 8/cod 8	9.12.18
12	69	20 +	IVS 2-1/	9.12.18
13	9610	4+25	cod 5/ cod 5	9.12.18
14	740	18 +20	cod 39/IVS 2-1	9.12.18
15	9602	15+20	IVS 1-110/ IVS 2-1	9.12.18
16	9616	15+ 29	IVS 1-110/IVS 1-110	9.12.18
17	303	9 +20	cod 8/9/ IVS 2-1	9.12.18
18	1278			9.12.18
19	609	9+20	cod 8/9/ IVS 2-1	12.1.19
20	567	20+33	IVS 2-1/ IVS 2-1	12.1.19



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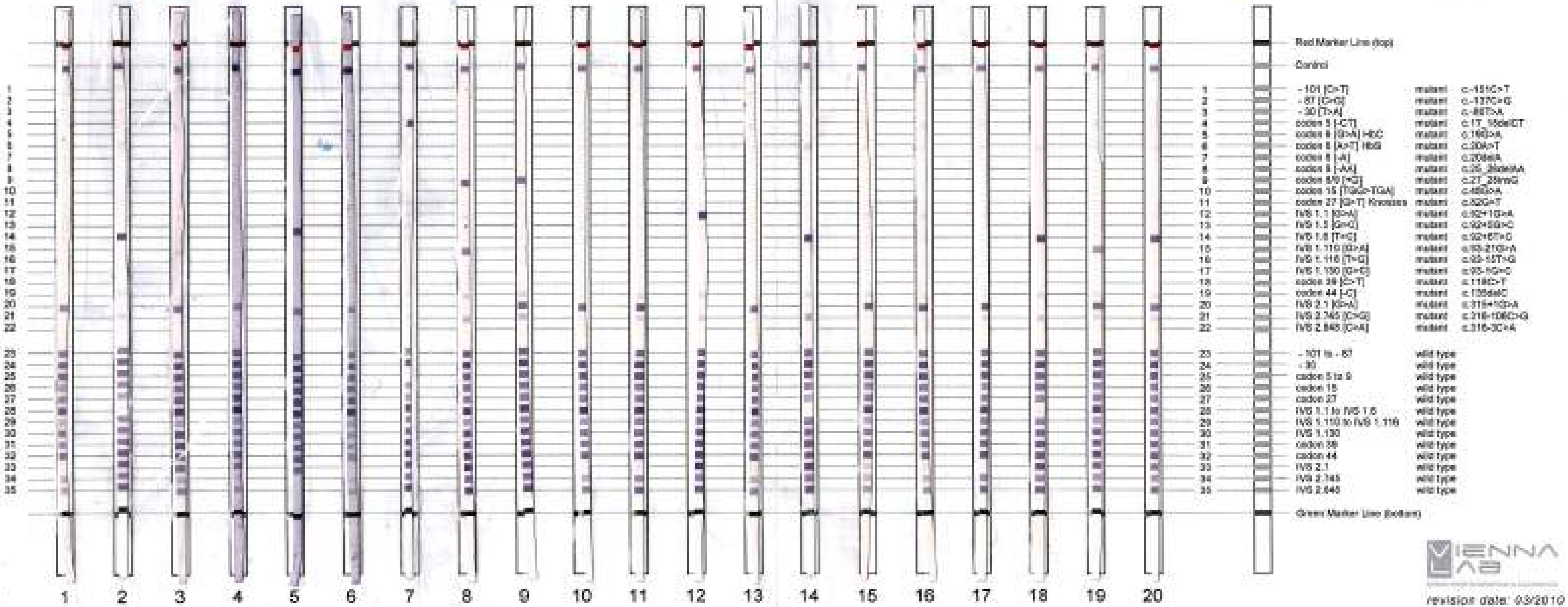
revision date: 03/2010

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT ______ use by ______

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	1337	20 + 33	IVS 2.1 / IVS 2.1	12.1.19
2	835	14 + 28	IVS 1.6 / IVS 1.6	12.1.19
3	1027	20 +	IVS 2.1 /	12.1.19
4	249	20 +	IVS 2.1 /	12.1.19
5	1000	13 + 20	IVS 1.5 / IVS 2.1	12.1.19
6	871	20 + 33	IVS 2.1 / IVS 2.1	12.1.19
7	987	4 + 25	cod 5 / cod 5	20.1.19
8	148	9 + 15	cod 8/9 / IVS 1.110	20.1.19
9	312	9 + 20	cod 8/9 / IVS 2.1	20.1.19
10	1271	20 + 33	IVS 2.1 / IVS 2.1	20.1.19
11	5712	20 + 33	IVS 2.1 / IVS 2.1	20.1.19
12	6299	12 +	IVS 1.1 /	20.1.19
13	9473	20 + 33	IVS 2.1 / IVS 2.1	20.1.19
14	9626	14 + 28	IVS 1.6 / IVS 1.6	20.1.19
15	9724	20 + 33	IVS 2.1 / IVS 2.1	3.2.19
16	9726	20 + 33	IVS 2.1 / IVS 2.1	3.2.19
17	9736	20 + 3	IVS 2.1 / IVS 2.1	3.2.19
18	1011	14 + 28	IVS 1.6 / IVS 1.6	3.2.19
19	593	15 + 20	IVS 1.110 / IVS 2.1	3.2.19
20	946	14 + 20	IVS 1.6 / IVS 2.1	3.2.19

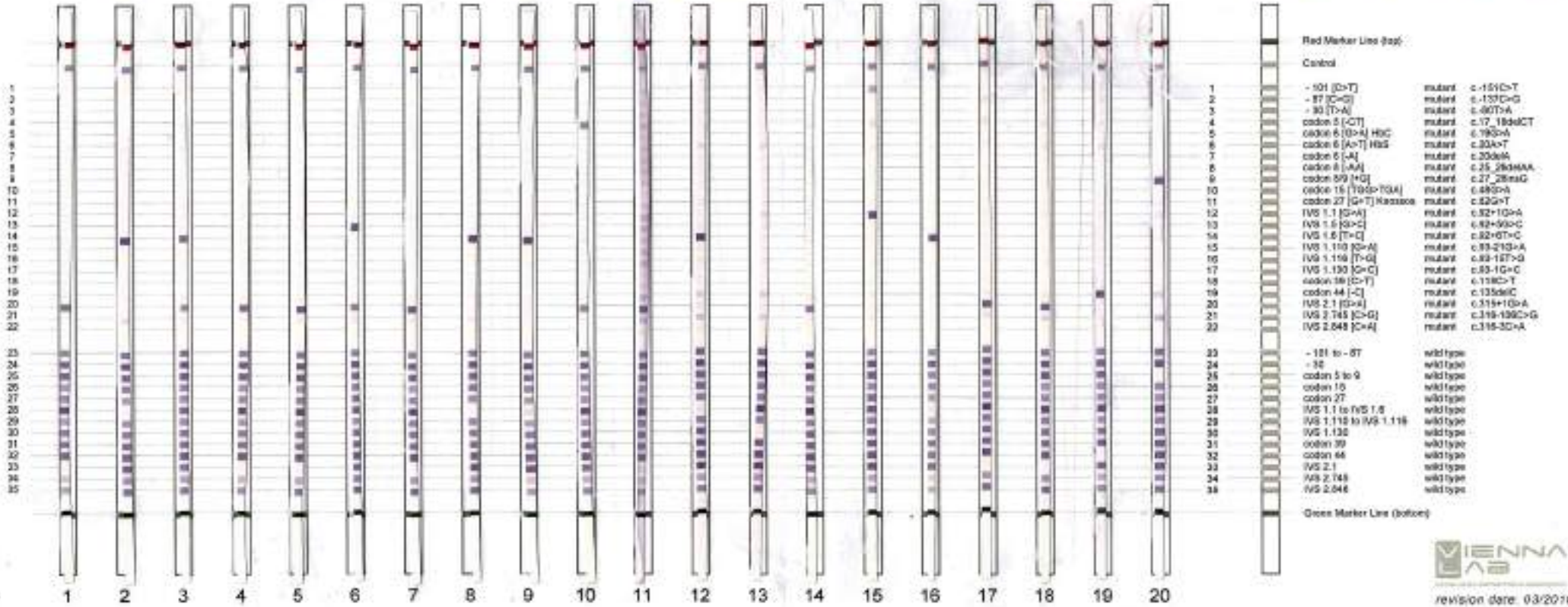


Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	1351	20+33	IVS 2.1/IVS 2.1	3.2.19
2	1343	14+28	IVS 1.6/IVS 1.6	3.2.19
3	8755	14+20	IVS 1.6/IVS 2.1	24.2.19
4	981	20+33	IVS 2.1/IVS 2.1	24.2.19
5	9370	20+33	IVS 2.1/IVS 2.1	24.2.19
6	178	13+20	IVS 1.5/IVS 2.1	24.2.19
7	9771	20+33	IVS 2.1/IVS 2.1	24.2.19
8	1155	14+28	IVS 1.6/IVS 1.6	24.2.19
9	1123	14+28	IVS 1.6/IVS 1.6	24.2.19
10	874	4+20	cod 5/IVS 2.1	24.2.19

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	886	20+33	IVS 2.1/IVS 2.1	3.3.2019
12	475	14+28	IVS 1.6/IVS 1.6	3.3.2019
13	242	30	IVS 1.130/	3.3.2019
14	1035	20+	IVS 2.1/	3.3.2019
15	9506	1+12	-101/IVS 1.1	3.3.2019
16	1065	14+28	IVS 1.6/IVS 1.6	3.3.2019
17	21	20+33	IVS 2.1/IVS 2.1	3.3.2019
18	431	20+33	IVS 2.1/IVS 2.1	3.3.2019
19	9321	19+32	cod 44/cod 44	10.3.2019
20	9865	9+25	cod 8/9/cod 9/9	10.3.2019



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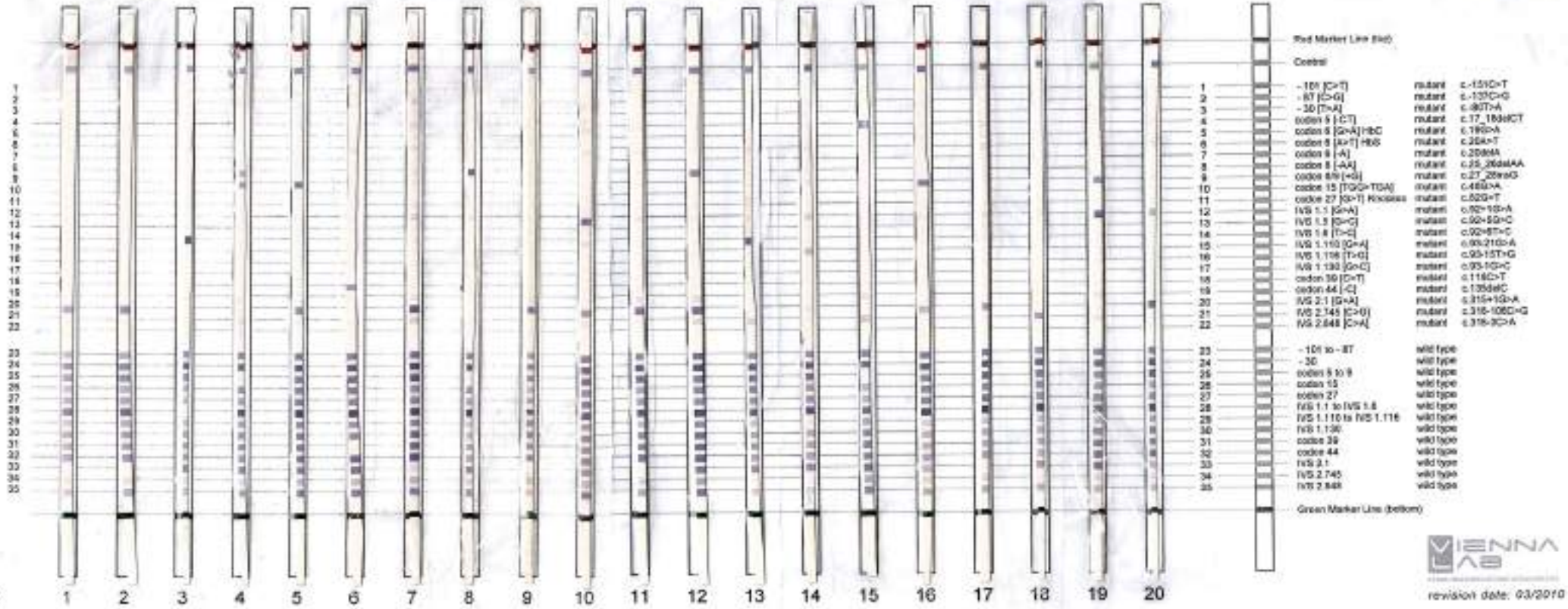
revision date: 03/2010

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	357	20 + 33	IVS 2.1/IVS 2.1	10.3.2019
2	769	20 + 33	IVS 2.1/IVS 2.1	10.3.2019
3	896	14 + 28	IVS 1.6/ IVS 1.6	10.3.2019
4	1038	8+9+25	cod 8/ cod 8/9	10.3.2019
5	287	9+20	cod 8/9/ IVS 2.1	10.3.2019
6	575	18 + 31	cod 39/ cod 39	10.3.2019
7	1239	20 + 33	IVS 2.1/IVS 2.1	17.3.2019
8	1194	8+ 25	cod 8/ cod 8	17.3.2019
9	1195	20 + 33	IVS 2.1/IVS 2.1	17.3.2019
10	1003	12 +20	IVS 1.1 / IVS 2.1	17.3.2019

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	1196	20 + 33	IVS 2.1/IVS 2.1	17.3.2019
12	223	8 +20	cod 8/ IVS 2.1	17.3.2019
13	1105	14 +	IVS 1.6/	17 3.2019
14	526	15+29	IVS 1.110/ 1.110	17.3 2019
15	937	4 + 25	cod 5/ cod 5	2.4.2019
16	271	9 + 20	cod 8/9/ IVS 2.1	2.4.2019
17	9555	20 +30	IVS 2.1/IVS 2.1	2.4 2019
18	57	21 +34	IVS 2.745/ 2.745	2.4.2019
19	1241	9 +12	cod 8/9/ IVS 1.1	2.4.2019
20	9317	12 +20	IVS 1.1/ IVS 2.1	2.4.2019

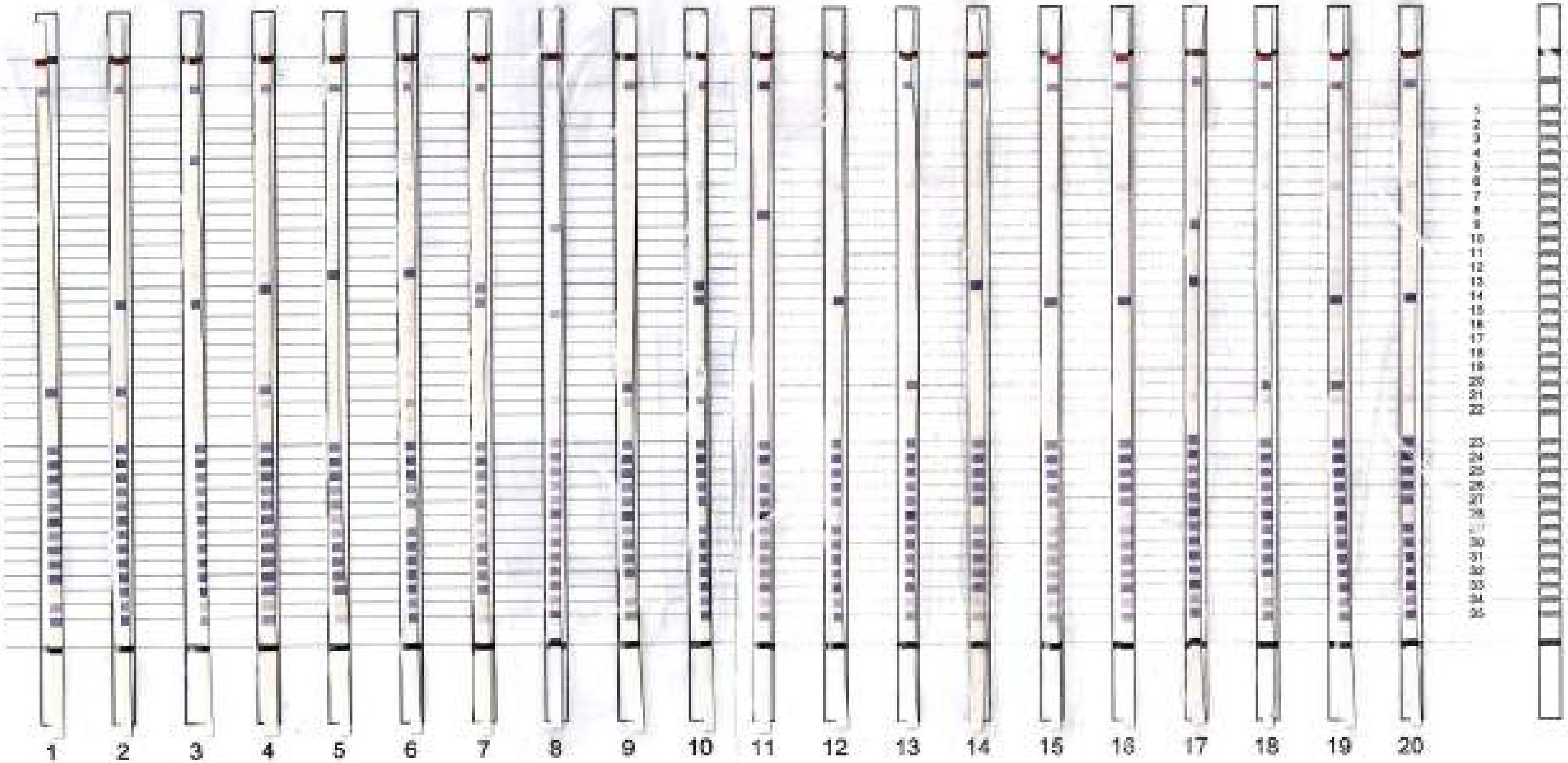


Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT ______ use by ______

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	443	20 + 33	IVS 2.1/2.1	2.4.2019
2	1307	14 + 20	IVS 1.6/2.1	2.4.2019.
3	1002	4 + 14	cod 5/IVS 1.6	14.4.2019
4	1001	20 + 13	IVS 2.1/ 1.5	14.4.2019
5	6298	12 +	IVS 1.1/	14.4.2019
6	2378	12 + 28	IVS 1.1/1.1	14.4.2019
7	502	13 + 14 + 28	IVS 1.5/1.6	14.4.2019
8	8932	9 + 15	cod 8/9 / 1.110	14.4.2019
9	360	20 + 33	IVS 2.1/2.1	14.4.2019
10	838	13 + 14 + 28	IVS 1.5/1.6	14.4.2019

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	9383	8 + 25	cod 8/cod 8	28.4.2019
12	797	14 + 28	IVS 1.6/1.6	28.4.2019
13	1036	20 +	IVS 2.1/	28.4.2019
14	799	13 + 28	IVS 1.5/1.5	28.4.2019
15	282	14 + 28	IVS 1.6/1.6	28.4.2019
16	744	14 + 28	IVS 1.6/1.6	5.5.2019
17	439	9 + 13	cod 8/9/IVS 1.5	5.5.2019
18	990	20 + 33	IVS 2.1/2.1	5.5.2019
19	870	14 + 20	IVS 1.6/2.1	5.5.2019
20	1155	14 + 28	IVS 1.6/1.6	5.5.2019



Red Marker Line (top)

Control

1	-101 [C>T]	mutant	c.-151C>T
2	-87 [C>G]	mutant	c.-137C>G
3	-30 [T>A]	mutant	c.-80T>A
4	codon 5 [-CT]	mutant	c.17_18delCT
5	codon 6 [G>A] HbC	mutant	c.19G>A
6	codon 6 [A>T] HbS	mutant	c.20A>T
7	codon 6 [-A]	mutant	c.20delA
8	codon 8 [-AA]	mutant	c.25_26delAA
9	codon 8/9 [+G]	mutant	c.27_28insG
10	codon 15 [TGG>TGA]	mutant	c.48G>A
11	codon 27 [G>T] Knossos	mutant	c.82G>T
12	IVS 1.1 [G>A]	mutant	c.92+1G>A
13	IVS 1.5 [G>C]	mutant	c.92+5G>C
14	IVS 1.6 [T>C]	mutant	c.92+6T>C
15	IVS 1.110 [G>A]	mutant	c.93-21G>A
16	IVS 1.116 [T>G]	mutant	c.93-15T>G
17	IVS 1.130 [G>C]	mutant	c.93-1G>C
18	codon 39 [C>T]	mutant	c.118C>T
19	codon 44 [-C]	mutant	c.135delC
20	IVS 2.1 [G>A]	mutant	c.315+1G>A
21	IVS 2.745 [C>G]	mutant	c.316-106C>G
22	IVS 2.848 [C>A]	mutant	c.316-3C>A
23	-101 to -87	wild type	
24	-30	wild type	
25	codon 5 to 8	wild type	
26	codon 15	wild type	
27	codon 27	wild type	
28	IVS 1.1 to IVS 1.6	wild type	
29	IVS 1.110 to IVS 1.116	wild type	
30	IVS 1.130	wild type	
31	codon 39	wild type	
32	codon 44	wild type	
33	IVS 2.1	wild type	
34	IVS 2.745	wild type	
35	IVS 2.848	wild type	

Green Marker Line (bottom)

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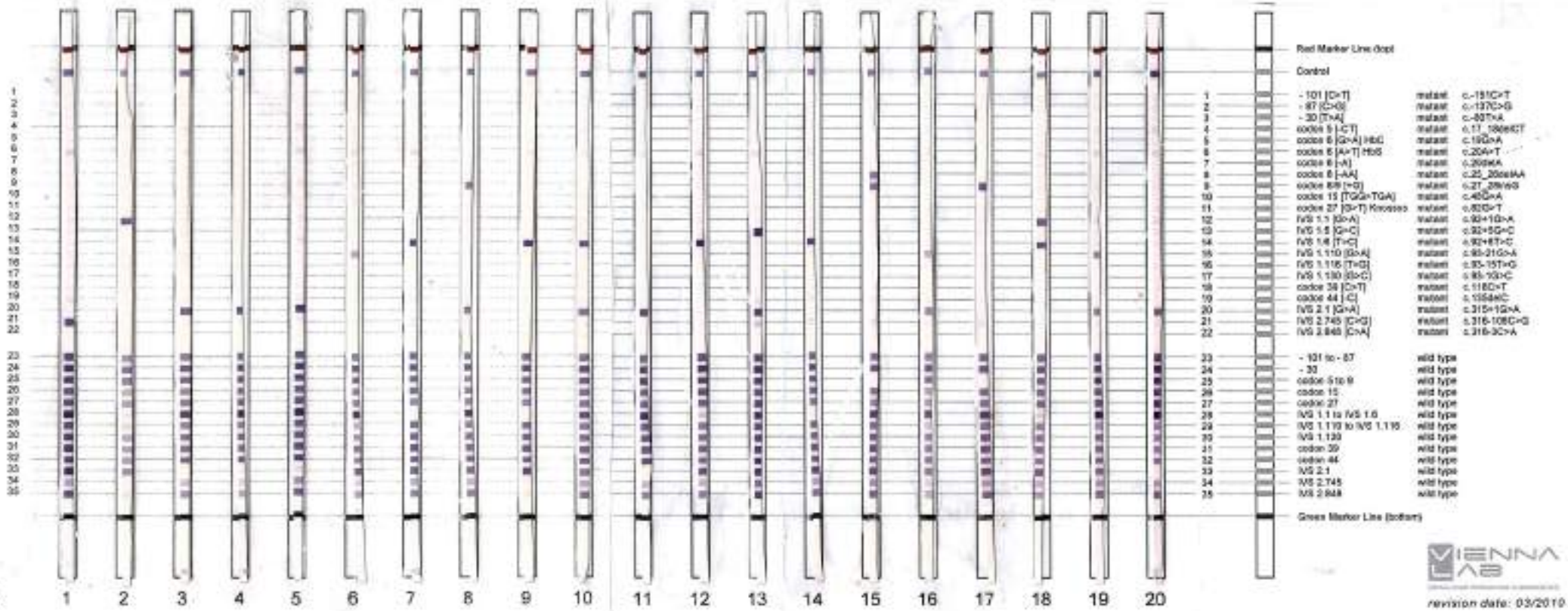
revision date: 03/2010

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	236	9+	2.745 ?	14.5.2019
2	9378	12 + 28	IVS 1.1/ IVS 1.1	14.5.2019
3	538	20 +33	IVS 2.1/IVS 2.1	14.5.2019
4	420	20 +33	IVS 2.1/ IVS 2.1	14.5.2019
5	37	20 +33	IVS 2.1/ IVS 2.1	14.5.2019
6	1057	15 +	IVS 1.110/	14.5.2019
7	476	14 + 28	IVS 1.6/ IVS 1.6	14.5.2019
8	936	9 + 20	cod 8/9/ IVS 2.1	14.5.2019
9	477	14 + 28	IVS 1.6/ IVS 1.6	28.5.2019
10	9674	14 + 20	IVS 1.6/ IVS 2.1	28.5.2019

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	16	20 + 33	IVS 2.1/ IVS 2.1	28.5.2019
12	9327	14 + 28	IVS 1.6/ IVS 1.6	28.5.2019
13	453	13 + 20	IVS 1.5/ IVS 2.1	28.5.2019
14	854	14 + 28	IVS 1.6/ IVS 1.6	28.5.2019
15	860	8 + 9 + 25	cod 8/ cod 8/9	28.5.2019
16	801	15 + 20	IVS 1.110/ IVS 2.1	28.5.2019
17	318	9 + 25	cod 8/9/ cod 8/9	10.6.2019
18	704	12 + 14 + 28	IVS 1.1/ IVS 1.6	10.6.2019
19	1024	15 + 20	IVS 1.110/ IVS 2.1	10.6.2019
20	655	20 + 33	IVS 2.1/ IVS 2.1	10.6.2019



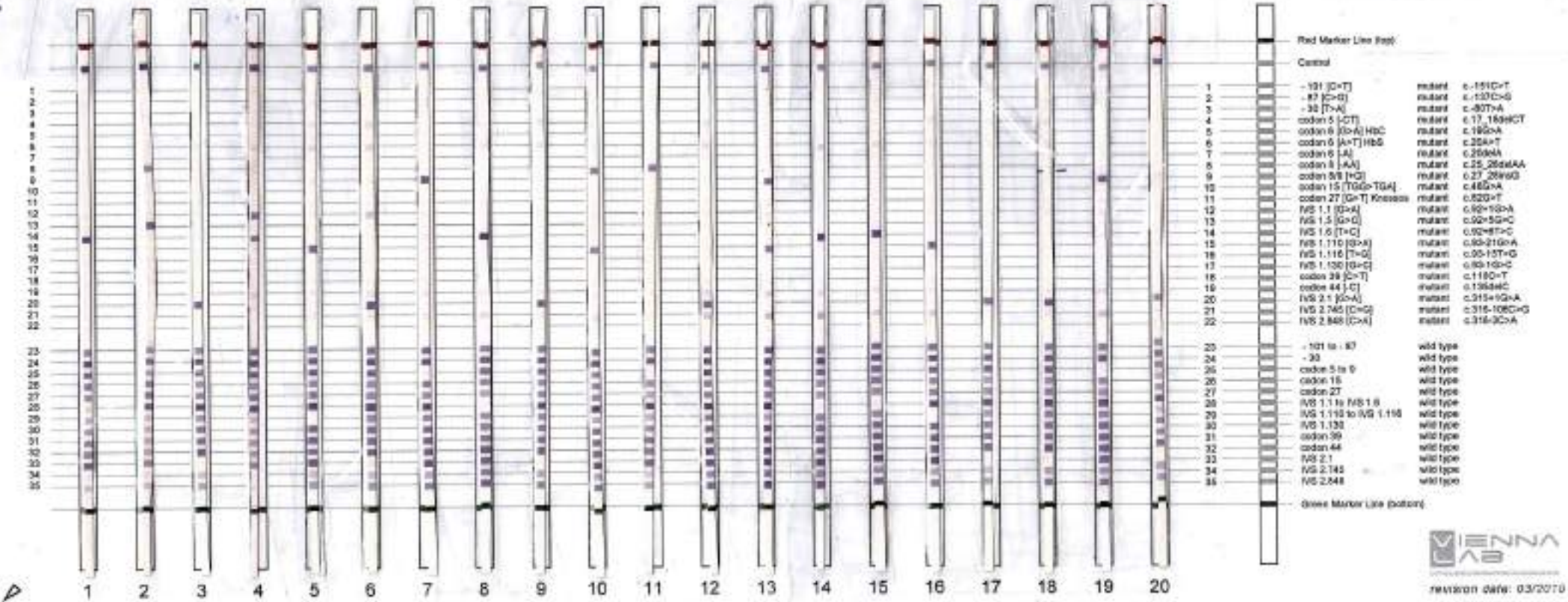
Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	5505	14 + 28	IVS 1.6/IVS 1.6	10.6.2019
2	65	8 + 13	cod8/IVS 1.5	10.6.2019
3	222	20 +33	IVS 2.1/ IVS 2.1	10.6.2019
4	5640	12 +14	IVS 1.1/ IVS 1.6	10.6.2019
5	704	15 +29	IVS 1.110/ IVS 1.110	24.6.2019
6	415	20 +33	IVS 2.1/ IVS 2.1	24.6.2019
7	9166	9 +25	cod 8/9/ cod 8/9	24.6.2019
8	797	14 +28	IVS 1.6/ IVS 1.6	24.6.2019
9	310	20 +33	IVS 2.1/ IVS 2.1	24.6.2019
10	174	8 + 15	cod 8/ IVS 1.110	24.6.2019

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11	8767	8 +25	cod 8/ cod 8	24.6.2019
12	375	20 +	IVS 2.1/	24.6.2019
13	285	9 + 15	cod 8/9/ IVS 1.110	8.7.2019
14	744	14 + 28	IVS 1.6/IVS 1.6	8.7.2019
15	7164	14 + 28	IVS 1.6/ IVS 1.6	8.7.2019
16	1034	15 + 29	IVS 1.110/ IVS 1.110	8.7.2019
17	990	20+ 33	IVS 2.1/ IVS 2.1	8.7.2019
18	508	20 +33	IVS 2.1/ IVS 2.1	8.7.2019
19	5864	9 + 25	cod 8/9/ cod 8/9	22.7.2019
20	8223	20 +33	IVS 2.1/ IVS 2.1	22.7.2019

IVS



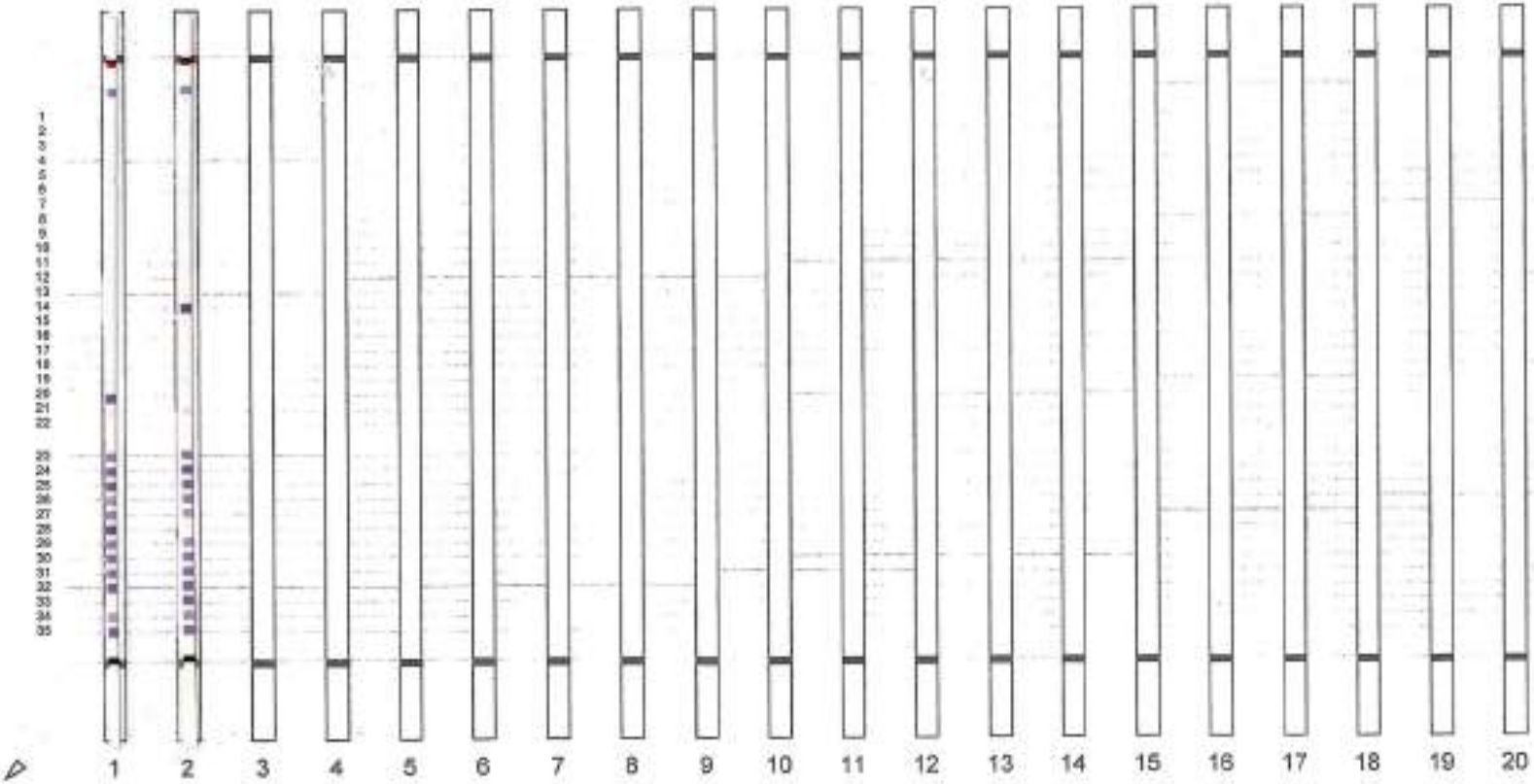
P

Collector ™ for β-Globin StripAssay ® MED

REF 4-130 LOT _____ use by _____

	Sample ID	Positive Lines	Genotype	Date/Operator ID
1	1098	20 + 33	IVS2.1/IVS2.1	22.7.2019
2	9973	14 + 28	IVS 1.6/IVS 1.6	22.7.2019
3				
4				
5				
6				
7				
8				
9				
10				

	Sample ID	Positive Lines	Genotype	Date/Operator ID
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				



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revision date: 03/2010

هەرێمی کوردستانی عیراق
وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
زانکۆی سلێمانی - کۆلێجی پزیشکی
بەشی نەخۆشیزانی

دەستنیشانکردنی بۆماوەیی و نەخۆشییەکانی پەیوەندیدار بە نەخۆشی بێتا سالاسیمیا لە باکوری ڕۆژهەڵاتی عیراق

دکتۆرانامەیەك پێشکەش کراوە بە بەشی نەخۆشیزانی و خوێندنی باڵای کۆلێجی پزیشکی\ زانکۆی سلێمانی وەك بەشێك لە پێداویستیەکانی بەدەستهێنانی بڕوانامەی دکتۆرا لە زانستی نەخۆشییەکانی خوێنی گەردی

لەلایەن

د. شه یما صالح أمین
دبلۆمی باڵا\ ماستەر لە زانستي نەخۆشییەکانی خوێن

سەرپەرشتیار

پڕۆفیسۆری یاریدەدەر د. سانە دلاوەر جەلال
دکتۆرا لة زانستي نەخۆشییەکانی خوێن

سەرپەرشتیاری یاریدەدەر

پڕۆفیسۆری یاریدەدەر د. کۆسار محمد علی
دکتۆرا لەنەخۆشییەکانی هەناوی و سنگ

خەرمانان 2720

پوختە

پاشخاتی زانستی: بیتا سالاسیمسا نەخۆشییەکی بۆماوەیی هیمۆغلۆبینی خوێنە، کە باوترین نەخۆشی تاك جینی یە لە هەمو جیهاندا، بە تایبەتی لە ڕۆژهەڵاتی دەریای سپی ناوەڕاست، کە عێراق و هەرێمی کوردستانیش دەگرێتەوە. لە ئاستی جیهاندا، تێکڕای ڕادەی بڵاوبونەوەی 4.4/10,000 لە دایك بووی زیندوو، لە گەلّ تێکڕای ڕادەی هەڵگری نەخۆشیەکە 1.5%. سیفاتە تایبەتمەندییەکانی ئەم نەخۆشییە بریتییە لە نا هاوسەنگی لە ڕێژەی نیّوان زنجیرەی α/β غلۆبین، دروستبونی خڕۆکەی سووری نا کاریگەر، کەم خوێنی دریژخایەن لە ئەنجامی تێکشکانی خڕۆکە سوورەکان، لە گەلّ زیادبوونی هەڵمژینی ئاسن لە ڕیخۆڵە. توندی نیشانەکانی نەخۆشی بیّتا سالاسیمیا جیاوازە بە شیّوەیەکی بەرفراوان، کە لە حاڵەتی هەست پێنەکراو دەست پێدەکات بۆ حاڵەتی زۆر توند تەنانەت کوشندە کە ئەمەش ڕەنگدانەوەی ئاستی نا هاوسەنگی نیّوان زنجیرەی α/β غلۆبین کە دیاریدەکرێت بە سروشتی گۆڕانکاری بازدان لە بیّتا جیندا. زیاتر لە 350 بازدان لەو جینەدا کە دەبێتە هۆی نەخۆشییەکە دیاریکراوە, کە شیّوازێکی جوگرافی و ڕەگەزی تایبەتی هەیە. نەخۆشی ئێسك، ماکەکانی جگەر و زراو، بەرزەپەستانی خوێنبەرەکانی سی، لەگەلّ چەندەها ماك لە سەر کوێرە ڕژێنەکان ئەمانە باوترین ماکەکانی نەخۆشییەکەن. چارەسەری نەخۆشییەکە بە شیّوەیەکی سەرەتایی بەندە لە سەر گواستنەوەی خوێن و چارەسەری لابردنی ئاسن لە خوێندا، هەروەها لابردنی سپلّ لە حاڵەتی تایبەتدا. سەرباری ئەوە، لەکەلّ زیادبوونی تێگەیشتن لە میکانیزمی بۆماوەیی و نەخۆشیزانی نەخۆشییەکە بووە هۆی پەیدابوونی ڕێگای چارەسەری نوێ.

ئامانجەکان : ئامانجی ئەم تویژینەوەیە بۆ دیاریکردنی جۆرەکانی بازدان لە جینی بیّتا غلۆبین لە هەردوو سالاسیمیای مامناوەند و سالاسیمیای گەورە لە سەنتەری سالاسیمیا لە سلێمانی، باکوری ڕۆژهەڵاتی عێراق. سەرباری ئەوە، هەڵسەنکاندنی نیشانەکانی نەخۆشییەکە لە سەر نەخۆشەکان و ڕێگا جیاوازەکانی چارەسەر کە لە سەنتەرەکەی ئێمەدا پیادەکراوە. ئامانجیکی دیکە، بۆ دیاریکردنی دووبارەبونەوەی جۆرەکانی

نەخۆشیەکانی پەیوەندیدار بە سالاسیمیاوە. لە کۆتاییدا، بۆ هەڵسەنگاندنی کاریگەری جۆری جینی لە سەر دروستبونی ماکەکانی نەخۆشییەکە و بەراوردکردنی جۆری جینی- جۆری سیفەتی نەخۆشی لە نێوان نەخۆشەکانی بێتا سالاسیمیا.

نەخۆش و ڕێگاکانی توێژینەوە: ئەم توێژینەوەیە ئەنجام دراوە لە سەر 242 نەخۆشی بێتا سالاسیمیا، کە پێکهاتووە لە 159 سالاسیمیای مامناوەند و 83 سالاسیمیای گەورە لە 162 خێزان کە تۆمارکراون و چارەسەر وەردەگرن لە سەنتەری سالاسیمیای سلێمانی. دیاریکردنی بازدانی بێتا سالاسیمیا بە ڕێگەی تەکنیکی هایبەردایزەیشنی پێچەوانە (reverse hybridization technique) و ڕیزبەندکردنی جینی ڕاستەوخۆ کراوە. هەروەها، سیفاتەکانی کلینیکی و نەخۆشی و چارەسەر، لەگەڵ گشت داتای تاقیگە، سەرباری ئەوە، (Dual Energy X-ray Absorptiometry scan) بۆ دەستنیشانکردنی ڕێژەی چڕی ئێسك و ئیکۆی دڵ بۆ دیاریکردنی بەرزە پەستانی خوێنبەرەکانی سی کە کۆکراوەتەوە لە ڕێگەی تۆمارکردن بە سیستمی داتای ئەلیکترۆنی لە ڕێگەی بەکارهێنانی پرسیارنامەیەکی گشتگیری داڕێژراو. سەرباری ئەوە، میژووی پزیشکی و پشکنینی جەستەیی تۆمارکراوە لە ڕێکەی ئاخاوتنی ڕاستەخۆ لەگەڵ نەخۆشەکان.

ئەنجامەکان: لە کۆی 22 بازدانی بێتا غلۆبین کە ڕێکخراوە لە 53 شێوازی جینی جیاواز دیاریکراوە، IVS II-1 (G>A) (35.7%) بە دوایدا ,(18.0%) IVS I-6 (T>C) و (8.5%) codon 8/9زۆرترین جار دووبارەبونەتەوە. بازدانی پێشوو باوترین بوو لە نێوان نەخۆشەکانی سالاسیمیای مامناوەند، بەڵام بازدانی دواتر باوترین بوو لە نەخۆشەکانی سالاسیمیای گەورە. بازدانە لە یەك چووەکان دیاریکرا لە % 76.3 نەخۆشەکان، لە % 62.9 لە ئەنجامی هاوسەرگیری خزمایەتی بووە. لە نێوان ماکەکانی پەیوەندیدار بە نەخۆشییەکە، نەخۆشی ئێسك باوترین بوو (% 66.9)، بە دوایدا نەخۆشیەکانی کوێرە ڕژێنەکانی (% 32.2)، ماکەکانی جگەروزراو (28.9%)، و بەرزەپەستانی خۆێنبەرەکانی سی (% 9.9). بە پێچەوانەوە، خوێن مەینی خوێنهێنەرەکان، دروستبونی خۆێن لە دەرەوەی مۆخی ئێسك، و بریینی لاق کەمترین جار

تێبینیکراوە. لە کۆتاییدا، چارەسەری هایدرۆکسی یوریا لە نەخۆشانی سالاسیمیای مامناوەند بووە ئەنجامی دابەزینی ڕێژەی فیّریتین لە پلازما، دووبارەبوونەوەی خۆیّن تێکردنی سالّانە، پێداویستییەکانی چارەسەری لابردنی ئاسن لە نیّوان نەخۆشەکانی بێتا سالاسیمیای مامناوەندی بەشداربوو.

دەرئەنجامەکان: ئەم تویّژینەوەی ئێستا، گەورەترین تویّژینەوەیە لە عیّراق و هەریّمی کوردستان لە سەر نەخۆشەکانی بێتا سالاسیمیا، ئاشکرایکرد کە بازدانەکانی β^0 سالاسیمیا باوترین بازدانە لە سالاسیمیای مامناوەندو سالاسیمیای گەورە کە ئەمەش جیاوازە لە تویّژینەوەکانی تری عیّراق و ووڵاتانی دەوروبەر. سەرباری ئەوە، سەرەڕای پەیڕەوکردنی چارەسەری پێوانەیی لە نەخۆشەکانی سالاسیمیا، هێشتا ڕێژەی ماکەکان زۆرە کە دیاریکرا بە ڕێژەی % 78.9 لە نەخۆشەکانی بەشداربوو، ڕێژەکە زیاتربوو لە نیّوان نەخۆشەکانی سالاسیمیای گەورە، و بە شێوەیەکی بەرچاو زیاتربوو لە نەخۆشەکانی $\beta^0\beta^0$ و $\beta^0\beta^+$ شێوازی جینی، و زیادبونی دروستبوونی ماکەکان لەگەلّ زیادبوونی تەمەن.

اقليم كوردستان ـ العراق
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التشخيص الجزيئي و الأمراض المرتبطة بمرض بيتا ثالاسيميا في شمال شرق العراق

رسالة مقدمة الى فرع علم الامراض والطب العدلي والدراسات العليا في كلية الطب\ جامعة السليمانية كجزء من متطلبات نيل شهادة الدكتوراه في علم امراض الدم الجزيئي

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بورد عراقي في أمراض الباطنية و الرئة

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الخلاصة

ألخلفية العلمية: بيتا ثلاسيميا هو مرض الهيموگلوبين الوراثي، وهو اضطراب الجين الأحادي الأكثر انتشارآ في جميع انحاء العالم، خصوصآ في منطقة البحر الأبيض المتوسط الشرقية، بما فيها العراق و منطقة كرستان- العراق. إن معدل الأنتشار المقدر هو 4.4/10,000 لكل ولادة حية و معدل الحامل المقدر 1.5٪. السمة المميزة للمرض تضمن اختلال التوازن في نسبة سلسلة ال α:β گلوبين، تكون الكريات الحمر الغير الفعالة، فقر الدم الإنحلالي المزمن، مع تعزيز امتصاص الحديد المعوي. الشدة السريرية لبيتا ثلاسيميا يختلف بنحو واسع تتراوح بين عدم ظهور الاعراض الى حالات شديدة أو حتى مميتة مما يعكس درجة اختلال توازن سلسلة الگلوبين التي تحددها طبيعة طفرات البيتا الجينية الأساسية. لقد تم تحديد أكثر من 350 الطفرت المسببة للمرض، ولها نمط جغرافي وأصل عرقي. إن أمراض العظام، مضاعفات الكبد والصفراء، ارتفاع ضغط الشريان الرئوي، اعتلالات الغدد الصماء المتعددة هي اكثر المضاعفات المرتبطة بالمرض و يعتمد العلاج التقليدي في المقام الأول على نقل الدم و علاج استخلاب الحديد, و كذلك، استئصال الطحال في حلالت محددة. بالإضافة الى ذلك، يؤدي الفهم المتزايد للأليات الجزيئية والفسيولوجية المرضية التي تحكم عملية المرض إلى تطوير مناهج علاجية جديدة.

الأهداف: كان الهدف من هذه الدراسة هو وصف طيف طفرات جينات البيتا گلوبين في كل من الأنماط الظاهرية للثلاسيميا الكبرى و الثلاسيميا الوسطى في مركز ثلاسيميا السليمانية في شمال شرق العراق. بالإضافة إلى ذلك، هدفت الدراسة إلى تقيم خصائص المرضى و طرق العلاج المختلفة التي يتم تنفيذها في مركزنا. هدفً أخر هو تحديد تكرر الأمراض المختلفة المرتبطة بالمرض و مقارنتها في كل من الطرز الظاهرية للثلاسيميا. و أخيرآ، إظهار تأثير النمط الجيني على تطور مضاعفات المرض و ترابط النمط الجيني - النمط الظاهري بين مرضى بيتا الثلاسيميا المسجلين.

المرضى و طرق البحث: هذه دراسة مقطعية أجريت على 242 مرضى بيتا ثلاسيميا، بما فى ذلك، 159 الثلاسيميا الوسطى و 83 الثلاسيميا الكبرى من 162 عائلة الذين تم تسجيلهم و تلقوا العلاج في مركز رعاية الثلاسيميا في السليمانية. تم الكشف عن طفرات البيتا الثلاسيميا عن طريق تقنية التهجين العكسي و التسلسل الجيني المباشر. كذلك تم تسجيل الخصائص السريرية و المرضية و العلاجية، مع جميع البيانات المختبرية، بلإضافة إلى فحص قياس أمتصاص الأشعة السينية مزدوج الطاقة لتقييم كثافة المعادن في العظام و تم تجميع تخطيط صدى القلب لتحديد ارتفاع ضغط الدم الرئوي من خلال نظام تسجيل طبي الكتروني باستخدام استبيان شامل مصمم. بالإضافة إلى ذلك، تم تسجيل التأريخ الطبي الكامل و الفحص البدني عن طريق إجراء مقابلات مباشرة مع المرضى.

النتائج: تم تحديد 22 طفرة بيتا گلوبين في هذه الدراسة، مرتبة في 53 من الأنماط الجينية المختلفة، منها IVS -II-1 (G>A) (35.7%) تليها IVS I-6 (T>C) (18.0%) و codon 8/9 (+G) (8.5%) أكثر شيوعآ. كانت الطفرة IVS-II-1 (G>A) هي الأكثر انتشارآ بين مرضى الثلاسيميا الوسطى، بينما كانت الطفرة codon 8/9 (+G) الأكثر انتشارآ في مرضى الثلاسيميا الكبرى. تم تحديد طفرات متماثلة الزيجوت في % 76.3 من المرضى، % 62.9 منهم نتيجة زواج الأقارب. وجدنا أن أمراض العظام كانت أكثرمضاعفات المرض تكرارآ % 66.9، يليها أمراض الغدد الصماء % 32.2، مضاعفات الكبد و الصفراء % 28.9، و ارتفاع ضغط الدم الرئوي % 9.9. على النقيض من ذلك، لوحظ تجلط الدم الوريدي، تكون خلايا الدم خارج النخاع، و قرحة الساق هي الاقل تكرارآ. و أخيرآ، كان إستخدام علاج الهايدروكسي يوريا بين مرضى ثلاسيميا الوسطى أدى إلى إنخفاض نسبة خزين الحديد في الجسم في المصل و اقله الحاجة لنقل الدم السنوي، و متطلبات العلاج بالأستخلاب بين مرضى بيتا ثلاسيميا المسجلين.

الأستنتاجات: الدراسة الحالية، الأكبر من العراق و أقليم كردستان على مرضى البيتا ثلاسيميا، كشفت بان طفرات β^0 ثلاسيميا كانت الطفرات الأكثر شيوعآ في بيتا الثلاسيميا الوسطى و الكبرى متميزة عن العراق و

الدول المجاورة. بالإضافة إلى ذلك، على الرغم من الإلتزام بالإرشادات العلاجية القياسية في مرضى الثلاسيميا، وجدنا نسبة عالية من المضاعفات التي تمت مواجهتها في 78.9 % من مرضى بيتا الثلاسيميا المسجلين، و كان المعدل اكثر تكرارآ بين مرضى الثلاسيميا الكبرى، و معدلات أعلى بشكل واضح في المرضى الحاملين $\beta^0\beta^0$ و $\beta^+\beta^0$ من الأنماط الجينية كما لوحظ إزدياد حدوث المضاعفات مع تقدم العمر.