NON-TRANSFUSION APPROACHES IN THE TREATMENT OF 
\( \beta \)-THALASSEMIA:

SPLENECTOMY AND 
PHARMACOLOGICAL AUGMENTATION OF FETAL HEMOGLOBIN

by

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A thesis submitted in conformity with the requirements
For the degree of Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto

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Non-Transfusion Approaches in the Treatment of β-Thalassemia: Splenectomy and Pharmacological Augmentation of Fetal Hemoglobin

ABSTRACT

Background
Regular transfusions allowing β-thalassemia patients to survive beyond childhood lead to toxic iron overload. Non-transfusion therapies may treat the anemia without introducing excess iron.

Hypothesis
Both splenectomy and pharmacological augmentation of fetal hemoglobin may allow survival without transfusions.

Methods
Pre- and post-splenectomy analysis was conducted on 10 β-thalassemia intermedia (TI) and 15 Hb E/β-thalassemia (E-thal) patients. Cohort comparisons of splenectomy vs. non-splenectomy were made in 32 TI and 108 E-thal patients.

Five infants were treated with sodium phenylbutyrate (SPB). Twelve children and adults were treated with SPB and hydroxyurea (HU).

Results
Five of the TI and ten of the E-thal patients responded following splenectomy. Mean hemoglobin was higher in the splenectomised E-thal cohort. No differences were seen in the TI cohorts.

No infants responded to SPB, but six children and adults responded to SPB and HU.

Conclusions
Splenectomy and augmentation of fetal hemoglobin may offer hope for β-thalassemia treatment.
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ABBREVIATIONS

5-AzaC = 5-Azacytidine
AraC = cytosine arabinoside
ALT = aminoaspartate transaminase
ANC = absolute neutrophil count
β-HCG = human chorionic gonadotropin
BFUe = burst forming unit-erythroid
BMT = bone marrow transplant
BRE = butyrate response element
CBC = complete blood count
CFUe = colony forming unit-erythroid
Cr = Serum Creatinine
Diff = white cell differential
EPO = erythropoietin
Hb = hemoglobin
Hb A = adult hemoglobin
Hb F = fetal hemoglobin
HPFH = hereditary persistence of fetal hemoglobin
HU = hydroxyurea
LCR = locus control region
MCV = mean corpuscular volume
MCH = mean cellular hemoglobin
MCHC = mean cellular hemoglobin concentration
MTD = maximum tolerated dose
pRBC = packed red blood cells
ABBREVIATIONS (cont'd)

PBS = phosphate buffered saline
PT = prothrombin time
RBC = red blood cell
RBS = reticulocyte buffered saline
rhEPO = recombinant human erythropoietin
SPB = sodium phenylbutyrate
STfR = serum transferrin receptor
TfR = transferrin receptor
WBC = white blood cell count
Chapter 1

Background

1.1 Pathophysiology of β-Thalassemia

β-Thalassemia encompasses a group of genetic mutations within the β-globin gene locus that either completely prevent (β⁰) or severely diminish (β⁺) the production of the β-globin chain. Therefore, patients with β-Thalassemia have an inability, or reduced ability, to make adult hemoglobin, or Hb A, which is composed of two α- and two β-globin subunits.

1.1.1 Hemoglobin Production

Hemoglobin is a tetrameric molecule comprised of two pairs of polypeptide subunits, each encoded by a different family of genes; the α-type genes found on chromosome 16 and the β-type genes found on chromosome 11. During the maturation of red blood cells, an accumulation of globin mRNA parallels a decline in the amount of other mRNA species, culminating in the nearly exclusive production of hemoglobin in maturing red blood cell precursors (Stamatoyannopoulos 1992), accounting for 90% of the red cell's dry weight.

There is generally an equal ratio of α- and β-type globin chains. It appears that the two clusters are co-ordinately but independently regulated as this balance is maintained in the absence of any apparent feedback between the two gene clusters (Huisman 1993).

1.1.1.1. Types of Hemoglobin

There are six major types of hemoglobin, formed by the interaction between two α-type subunits and two β-type subunits, which are synthesized in erythropoietic cells. The form of α- or β-type sub-unit produced is determined by co-ordinate switches that occur among the α- and β-globin gene clusters. Figure 1.1 depicts the developmental changes in globin chain production.

Hematopoiesis occurs in the yolk sac during the embryonic period; moves to the liver at about five weeks; and moves to the spleen and bone marrow at about 20 weeks. Thereafter, the relative amount of hemoglobin produced in the spleen decreases, and by birth, it is produced primarily in the bone marrow.
In the embryonic period, the primary β-type globin is ε-globin. This combines with either ξ- or α-globin to form Hb Gower 1 (ξε2ε) or Hb Gower 2 (αε2ε). Small amounts of γ-globin are also produced at this point, combining with the α-type globin chains to form Hb Portland (ξεγ2) and fetal Hb, or Hb F (αγε). There are two γ-globin chains, Aγ and Gγ, which differ in only one amino acid, at codon 136, which is alanine in Aγ and glycine in Gγ (Huisman 1993). The two forms of Hb F are found in different ratios throughout development. In a normal newborn, the Aγ:Gγ ratio is generally 1:2, while in the adult it is 3:7 to 4:6, but the ratio is much more variable (Huisman 1993, Wood 1993).

Both ξ- and ε-globin production immediately decrease and by ten weeks gestation they are no longer produced. At this point, the two main globin chains being produced are α- and γ-globin and Hb F is the main form of hemoglobin produced. δ-globin, a β-type globin is produced in minor amounts beginning at 32 to 36 weeks gestation and combines with α-globin to make Hb
A₂, which is produced in small amounts (1 - 3.5%) throughout life. β-globin expression begins when ε-globin expression stops and continues to increase until, at birth, it accounts for approximately 20% of the β-type globin production. The α-globin and β-globin chains combine to form Hb A. Hb F, which accounts for approximately 80% of total hemoglobin at birth (in a healthy term infant), gradually decreases to 1 to 2% by 12 months of age and is restricted to 0.1 to 7.0% of the red cells (Bard 1975, Bunn 1986). These cells, termed F-cells, contain about 20% Hb F. It is proposed that F-cell formation is regulated during the differentiation of BFU-e and CFU-e during early erythropoiesis; individual colonies derived from BFU-e include both cells than contain Hb F and cells that do not, while CFU-e colonies are composed either of F-cells or non F-cells (Umemura 1988). While the earliest red cell progenitors appear to be able to develop either along a low or high F pathway, in normal conditions, most cells follow the low F pathway (Pearson 1996). Stamatoyannopoulos (1985) proposed a model in which F-cells represent the progeny of erythroid progenitor cells that had undergone an 'accelerated' pathway of erythroid differentiation. This accelerated pathway was said to be due either to the skipping of cellular divisions and/or a reduction in the cycling time of cells in each step; and, results in the formation of F-cells by preventing the completion of changes in chromatin structure (or methylation) that occur during maturation/differentiation of erythroid progenitors and inactivate the γ-globin gene. When erythroid stress is increased, there is a depletion of late progenitor pools, leading to an increase in the proportion of cells that undergo accelerated maturation/differentiation and the proportion of F-cells is thus increased (Stamatoyannopoulos 1987, Stamatoyannopoulos 1985). The relative level of Hb F is greater in acute states of erythroid stress and expansions (i.e., treatment with cytotoxic agents) than in chronic states of erythroid stress and expansion (i.e., β-thalassemia) (Blau 1993, Stamatoyannopoulos 1985). Patients with increased Hb F can exhibit either an increase in the number of F-cells and/or an increase in the amount of Hb F per F-cell. For example, patients with increased levels of Hb F due a to polymorphism at the Xmn 1 site, the number of F-cells are increased but these cells continue to have approximately 20% Hb F per cell (Jane 1998).

1.1.1.2. Control of Globin Chain Expression
Developmental changes in hemoglobin production occur in all vertebrate species but the fetal to adult switch occurs only in ruminants and primates (Wood 1993, Wood 1983). It is believed that there are three factors responsible for control of globin expression in humans: (1) cis-acting sequences within the gene that regulate the switches; (2) distal sequences that act as enhancers; and (3) trans-acting proteins that bind to the regulatory elements (Jane 1998).
The presence of nuclease-hypersensitive sites in a region of DNA supports the presence of regulatory elements in the region because regulatory elements are devoid of nucleosomes and hence are hypersensitive to nuclease (Stamatoyannopoulos 1992). The nucleosomes in the region have been displaced or destabilized by the binding of trans-acting factors.

A region of four DNase hypersensitive sites is located upstream of the β-globin gene locus, and is termed the locus control region (LCR). The LCR is an absolute requirement for expression of all of the genes in the β-globin locus. Evidence of this is shown by both erythroleukemic cells and transgenic mice, which exhibit increased globin production when the regions around each hypersensitive site are linked, alone, or in combination, to the β-globin gene (Stamatoyannopoulos 1992). Furthermore, Hispanic γδβ-thalassemia removes parts of the LCR, but leaves the entire β-globin cluster intact. Yet none of these genes are functional, proving the LCR is needed for expression of genes within the β-globin gene locus (Driscoll 1989, Ley 1991). The LCR determines the level of gene expression by altering the frequency and duration of transcription as well as the rate of transcription (Grosveld 1998). There appears to be competition between the genes on the β-globin locus, as the expression of one gene can decrease the expression of another; the genes closer to the LCR have stronger suppressive effect on the more distally located genes (Grosveld 1998, Stamatoyannopoulos 1992). Furthermore, the β-globin locus changes shape as different genes are expressed (Jane 1998) since the LCR can only interact with one gene at a time. Therefore regulation appears to act through direct interaction between the LCR and gene (Grosveld 1998).

Regulation of globin gene transcription is regulated, in part, by proteins that bind to specific motifs within the cis-acting regulatory elements of the globin genes, which include promoters, enhancers, and silencers (Stamatoyannopoulos 1992). Mutations within the proximal γ-globin gene promoter can lead to HPFH, highlighting this importance of the promoter in silencing or activating the γ globin (Li 1998).

Postsynthetic modifications to DNA and chromatin may also play a role in the coordination of globin gene expression. Methylation of cytosine residues by DNA methyltransferase occurs shortly after replication. A general correlation between gene expression and the frequency of DNA methylation appears to play a role in regulatory regions (Stamatoyannopoulos 1992).
1.1.1.3. Pathophysiology of Hemoglobin Production in β-Thalassemia

Approximately 200 different mutations leading to the β-thalassemia phenotype have been identified. There are several polymorphisms detectable with restriction endonucleases in the β-globin locus. Five are in the 5’ subhaplotype, while two are in the 3’ subhaplotype (which contains the β-globin gene). Each of these haplotypes tends to be associated with a few specific mutations (Flint 1993).

Patients with β-thalassemia either do not produce any β-globin chains, or produce them at a diminished rate. In heterozygotes, Hb A₂ is elevated about two-fold and mean cellular hemoglobin (MCH) is about 70% of normal (Bunn 1986). In most β-thalassemia homozygotes, however, absolute Hb A₂ does not appear to be increased. This is because, although, the proportion of Hb A₂ is usually increased to 2.3 to 3.0%, the marked microcytosis and the preferential survival of cells containing high levels of Hb F and low levels of Hb A₂, reduces the amount of absolute circulating Hb A₂ (Bunn 1986). As in patients with sickle cell anemia, patients with β-thalassemia show a slower fall off in the production of Hb F after birth than normal term infants (Pearson 1996). Patients tend to have higher than normal levels of Hb F throughout life, but this generally results from the mobilization of cells into the high F pathway, not γ-globin gene activation, and is insufficient to prevent anemia and hemolysis (Pearson 1996). F-cells also appear to have preferential survival over those with no Hb F (Wood 1983).

1.1.2. Red Cell Metabolism

A healthy 75 kg adult produces about $2.6 \times 10^6$ reticulocytes every second, replacing 0.8 – 1.0% of the circulating pool of red cells daily (Cazzola 1992). These cells have an average lifetime of 120 days and are then destroyed; the parts are either recycled or eliminated.

1.1.2.1. Normal Red Cell Maturation

The most primitive red cell progenitors are totipotent stem cells, from which are derived lymphoid and myeloid pluripotent stem cells. The myeloid stem cells are capable of differentiating into megakaryocyte, granulocyte/macrophage or erythrocyte precursors. Growth factors (i.e., chemical factors) stimulate the production of specific cell types. The kit ligand, interleukin-3, and granulocyte-macrophage colony-stimulating factor appear to be the predominately active compounds when BFU-e are derived from the committed stem cells.
The development of CFU-e from BFU-e takes about six to seven days and is extremely erythropoietin (EPO) sensitive. EPO, secreted by the liver and kidneys, is important in the formation of red cells. Proerythroblasts (early committed red cell precursors) are formed after one to two days.

The total maturation time of red-cell precursors (from proerythroblast to reticulocyte) is seven days. The first four days involve cell division and the last three entail cellular maturation and hemoglobin synthesis. The reticulocytes remain in the bone marrow for another 24 hours and then remain in the periphery for 24 - 48 hours before maturing into red cells. Therefore, the total time from proerythroblast to mature red cell is approximately nine to ten days. This time can be shortened by reducing the inter-mitotic interval and/or by skipping some mitotic divisions.

During cell division, the proerythroblasts divides, the chromatin condenses and the ribosomes increase in number. The cells transform to basophilic erythroblasts and then to polychromatophilic erythroblasts; each proerythroblasts produces 16 polychromatic erythroblast daughters. As the cells mature into orthochromatophilic erythroblasts, the mitochondria and RNA are lost, the nucleus shrinks, and the full complement of hemoglobin is synthesized. Once the nucleus is extruded from the cell it is considered a reticulocyte. The reticulocytes are released from the bone marrow into the blood stream, where they develop into mature red blood cells that are biconcave discs with a diameter of about 7.5 μm and a circumferential thickness of 2.5 μm. This occurs in 24 to 48 hours. Up to 10% of developing red cells are lost during maturation in normal adults, occurring mainly at the late orthochromatophilic erythroblast stage (Poottrakul 2000) and 1% of mature red cells are lost every 24 hours through senescence. These are replaced by the maturing reticulocytes, which comprise approximately 1% of total red cells.

1.1.2.2. Normal Red Cell Destruction
Eighty to 90% of normal red cell destruction is extravascular and occurs within the macrophages of the spleen; the hemoglobin is not released into the plasma. In the 10 to 20% that are destroyed within the circulation, the globin is hydrolyzed into its component amino acids which are then reused in protein synthesis; the iron is released from the heme, combines with transferrin, returns to the bone marrow and is reused in erythropoiesis; and iron free heme is metabolized to biliverdin.
Biliverdin is the first product of heme destruction, formed by cleavage of the α-methane bridge. Biliverdin is then reduced to bilirubin, which is complexed with serum albumin and transported to the liver, where it is conjugated by glucuronate. This conjugation, in which sugar residues are added to propionate side chains, leads to the solubilization of bilirubin. The conjugated bilirubin is secreted into the bile and then excreted. In normal adults, 95% of the measurable bilirubin in plasma is unconjugated; while, little or no unconjugated bilirubin is found in bile.

1.1.2.3. Pathophysiology of Red Cell Metabolism in β-Thalassemia

McCurdy described reduced red cell survival time in patients with sickle cell disease in 1969 (McCurdy 1969). Vigi et al. (Vigi 1969) later compared 13 patients with homozygous β-thalassemia, 3 patients with heterozygous β-thalassemia, and 5 normal controls to determine if a correlation between red cell survival and excess α-globin chain synthesis exists. In the control patients (α/non α ratio: 0.98 to 1.03), the $^{51}$Cr half-life was 28 to 31 days (equivalent to a red cell life span of about 120 days). The heterozygous β-thalassemia patients (α/non α ratio: 1.3 to 1.7) and homozygous β-thalassemia patients (α/non α ratio: 1.9 to 3.5) had $^{51}$Cr half-lives of 18 to 25 and 7 to 17 days respectively. Vigi reported an inverse relationship between α/non α globin chain synthesis ratios and red cell survival ($r=0.9; P<0.01$). In 14 patients with Hb E/β-thalassemia, a correlation of 0.79 was observed between hemoglobin and red cell $^{51}$Cr half-life red cell survival time (Wasi 1985).

In 1971, Shahid described 15 normal patients as having a mean $^{51}$Cr half-life of 28.9 ± 2.6 (25 to 36) days. Shahid tested both homologous and autologous red cell survival in 15 thalassemia patients. Autologous red cell survival was the same for both transfusion-dependent (n=7) and transfusion-independent (n=7) patients; 15.7 ± 3.3 days in transfused patients vs. 15.7 ± 3.0 days in untransfused patients (P=NS). However, there was a significant difference in homologous red cell survival between these two groups. In regularly transfused patients, the mean homologous red cell survival (13.0 ± 7.0 days) was slightly lower than autologous red cell survival. Compared to the controls, homologous red cell survival was significantly lower in the transfused patients (P<0.01), while homologous red cell survival in transfusion-independent patients was 26.2 ± 3.8 days, which was similar to that seen in the normal controls (P>0.1). Neither hemoglobin concentration nor transfusion requirement correlated with autologous red cell survival. However, homologous red cell survival was positively correlated with hemoglobin concentration and negatively correlated with transfusion requirement. Three of the patients
studied underwent a second red cell survival study, 23 to 72 months after a splenectomy. There was no significant change in autologous red cell survival \[14.1 \pm 3.4 \text{ (8.3 - 20) days prior to splenectomy, to } 15.3 \pm 3.5 \text{ (9 - 21) days after splenectomy}.\] But, homologous red cell survival increased from \[12.7 \pm 8.1 \text{ (3.6 - 29) days to } 28.7 \pm 1.5 \text{ (26 - 29) days.}\] These three patients had an increase in baseline hemoglobin concentration and a decrease in transfusion requirement, both of which were shown to affect homologous red cell survival. Shahid proposed that the more severely affected patients had reduced homologous red cell survival times due to iso-immunization from multiple transfusions. This, caused by foreign red cell antigens found in transfused blood, may have led to a destruction of the homologous cells (Shahid 1971). In studies of hypertransfusions protocols in patients with \(\beta\)-thalassemia, Piomelli calculated a weekly decline in hemoglobin concentration, following transfusions. From this, Piomelli determined that transfused red blood cells have an approximate life span of 12 weeks (Piomelli 1969).

Suzuki performed the initial study using biotin to label red cells in 1987. He utilized double-labeled rabbit erythrocytes with both biotin and \(^{14}\)C-cyanate. Two rabbits were labeled with both biotin and \(^{14}\)C-cyanate, and a third rabbit was labeled only with \(^{14}\)C-cyanate. A life span of approximately 53 days was seen in all three rabbits. An initial fear was that biotinidase, an enzyme that cleaves biotin from some peptides and is found in serum, could cleave the biotin from the red blood cells; comparison of the survival of biotin label versus \(^{14}\)C-cyanate label was able to dispel this fear. Over 18 days, the levels of each label decreased in parallel. Biotin labeled cells were isolated from the whole blood samples using avidin-biotin-gelatin beads, which specifically bind biotin labeled red blood cells. The ratio of \(^{14}\)C/Hb in the unfractioned red cell population decreased with time from infusion as the labeled cells died and new, unlabeled cells were made; while, the ratio of \(^{14}\)C/Hb in the biotinylated samples (those isolated through the avidin-biotin-gelatin beads) remained constant through out the survival study, indicating that the two labels were being lost at equal rates and that biotin is a good substitute for the radio-label method (Suzuki 1987).

Hoffmann-Fezer et al. further demonstrated the usefulness of biotin labeling in determining red cell survival through in vivo and in vitro labeling of red blood cells in mice. Two to three infusions of 0.75 - 1.0 mg biotin were able to label 100% of the cells in vivo. This label decreased to 86.4 \(\pm\) 3.2% on day 36. In the in vitro labeled animals, the percent label on day 36 was 67.3 \(\pm\) 7.3%. The mean half-life, determined through linear regression, was 23.6 days.
(range: 22.8 – 25.6 days); through logarithmic regression it was 8.6 (8.0 – 12.1) days. Label was <1% on day 46 in all animals. The in vitro studies gave a mean half-life of 24.0 ± 0.7 (21.8 to 26.0) days by linear regression and 9.1 ± 0.7 (6.9 – 11.0) days by logarithmic regression (Hoffmann-Fezer 1993).

Mock et al. confirmed the use of biotin to determine in vivo red cell survival in humans. He compared the survival determined with biotin to that determined with $^{51}$Cr in 10 controls. The average life span with biotin was 103 ± 8 days, while that determined by $^{51}$Cr was 116 ± 16. The biotin survival was slightly, but significantly, reduced indicating that biotin labeling does induce minimal damage. In one patient, maximum life span was determined as 133 to 147 days (Mock 1999).

1.1.2.4. Measurement of Ineffective Erythropoiesis
1.1.2.4.1. Serum Transferrin Receptor Concentration

The transferrin receptor (TfR), comprised of two identical subunits of 95,000 daltons each, controls the flow of transferrin iron into cells by enabling transferrin to enter the cell and release its iron (Huebers 1990). The total number of cellular receptors determines the amount of iron uptake; the cells with the highest concentration of TfR include rapidly dividing cells, hemoglobin-synthesising cells, and the placenta; while, iron-replete cells show a reduction in TfR to prevent the accumulation of excess iron (Cook 1993, Huebers 1990). Sensitive immunological assays can detect TfR in circulation. This serum transferrin receptor (STfR) is intact receptor that has been cleaved from the tissues and cells. STfR are derived from abnormal immature erythroid cells destroyed before reaching the reticulocyte stage, as well as from maturing reticulocytes (Huebers 1990), causing STfR concentration to be increased in ineffective erythropoiesis.

Tissues contribute to STfR in the relative proportion that the cellular TfR are distributed. As ferrokinetic studies have shown that at least two thirds of TfR are located on erythroid tissues (with 80% located in the erythroid marrow), it is reasonable to assume that two thirds of the STfR reflect production from erythroid tissues and that the erythroid marrow is the main source of STfR, so elevated levels of STfR provide a valuable measure of increased erythropoiesis (Cazzola 1992, Cook 1993, Huebers 1990).

In a study of 148 patients, Huebers et al. (1990) showed a correlation of 0.86 (P < 0.001) between STfR concentration and ferrokinetic measurements of erythropoiesis. The study
In a study of 148 patients, Huebers et al. (1990) showed a correlation of 0.86 (P< 0.001) between STfR concentration and ferrokinetic measurements of erythropoiesis. The study population included normal subjects, and patients with hypoplastic erythropoiesis, hyperplastic erythropoiesis, altered iron status, and malignancies; the relationship between STfR and erythropoiesis, as measured by erythron transferrin uptake, was consistent between the disease and control states studied. Erythron transferrin uptake measures the number of iron-bearing molecules taken up by tissue receptors per unit time (Huebers 1990). Also consistent across the levels of erythropoietic activity was the fraction of total receptor that was represented by the STfR, which was about 6%.

72 patients with Hb E/β-thalassemia showed a mean increase in STfR concentration of 8.5 times normal; 8.3 ± 1.3 mg/L in the normal controls vs. 70.0 ± 22.4 mg/L in the patients with Hb E/β-thalassemia (P<0.001).

Singhal et al. determined STfR concentrations in 34 Hb SS patients at three age points (1-3, 4-6, and 7-9 years). STfR concentration tended to rise with age, and the increase within individuals correlated to increases in reticulocytes (r=0.38, P=0.017) and to decreases in Hb F (r=0.51, P=0.004) (Singhal 1993).

1.1.2.4.2. Serum Erythropoietin Concentration

EPO is a 35 kD glycoprotein hormone secreted by the kidney that induces the formation of red blood cells by stimulating proliferation, differentiation and maturation of the cells (Papayannopoulou 1976), derived from a single gene on chromosome 7. EPO gene activation is linked to hypoxia, or reduced hemoglobin concentrations, presumably through ‘the hypoxic-responsive element’ positioned in the 3' tail of the gene (Beck 1991), and EPO is the main regulator of red cell production (Erslev 1995). High levels of EPO may shorten the marrow transit time of erythroblasts, leading the release of immature red cells, such as nRBC (Erslev 1995, Finch 1970). A feedback mechanism exists between EPO and hemoglobin concentration. EPO is triggered by low hemoglobin concentration (hypoxic conditions), the increase in serum EPO levels leads to increased formation of red blood cells, and hemoglobin concentration increases, alleviating the hypoxic conditions which switches off the EPO gene activation. However, in patients with β-thalassemia erythropoiesis is ineffective and hypoxic conditions are not alleviated and EPO levels remain elevated. Therefore, the breakdown of the natural feedback mechanism is indicative of the ineffective erythropoiesis seen in β-thalassemia.
1.1.3. Clinical Manifestations of β-Thalassemia

Anemia is the main cause of morbidity and mortality worldwide (Weatherall 2000). The first thalassemic patients to be described in the literature were by Cooley who described seven patients with hemolytic anemia, splenomegaly, and distinct Mongolian features (Cooley 1925, Cooley 1927). Thalassemia or Cooley's anemia was first thought to be restricted to people living in the Mediterranean (Italians, Greeks, Syrians, and Armenians) (Whipple 1936), but it has since been shown that it is found in regions around the world that are, or were, endemic for malaria (Flint 1993). Haldane was the first to propose that the increased frequency of hemoglobinopathies in these regions may be due to a resistance to malaria seen in heterozygotes (Weatherall 1997, Weatherall 1998).

Most of the problems in β-thalassemia are caused by the imbalance in globin chain synthesis, as opposed to the under hemoglobinisation of red cells (Nathan 1966). Excess α chains can liberate iron (Scott 1993) and precipitate in the red cell precursors, thereby preventing normal maturation and decreasing red cell survival (Weatherall 1996). These precipitates were first reported to form spontaneously in vivo in red cells from β-thalassemia patients by Fessas in 1963 (Fessas 1963). He observed inclusions that differed to hemosiderin granules, but displayed the staining properties of hemoglobin.

Ineffective erythropoiesis is caused by cell death in the marrow and decreased survival is caused by the impaired deformability of the more rigid cell membrane leading to hemolysis and rapid removal of cells from circulation. The accelerated apoptosis is found to increase with erythroid maturation, with little or no apoptosis seen in pronormoblasts or basophilic normoblasts (Pootrakul 2000). This is expected, as the amount of α-globin precipitate increases as hemoglobin production increases. Fessas reported the appearance of inclusion bodies in the more mature red cell precursors of the bone marrow, but only fine, granular particles distributed through the cytoplasm of earlier progenitors (Fessas 1963). Presumably, this particulate matter represented excess α-chains that had not yet accumulated to the extent that precipitation occurred.

Due to the anemia, erythropoiesis is increased up to 10-fold; however, owing to the underlying mutation(s), this erythropoiesis may be up to 95% ineffective (Olivieri 1999), leading to a hypertrophy of ineffective bone marrow (Weatherall 2000). Ferrokinetic techniques have
estimated that the rate of erythroid precursor proliferation in severely anemic patients is ten to 20 times the basal level; this is higher than those seen in any other human disorder (Cazzola 1995). The hypertrophy leads to the typical ‘thal facies’ with prominent frontal bossing, prominent cheek bones, and protruding upper jaw due to the expansion of marrow in the skull and facial bones (Bunn 1986). Other effects of bone marrow hypertrophy include osteoporosis or osteopenia due to bone marrow expansion in the long bones, which causes rarefication; hepatosplenomegaly; and masses due to extramedullary hematopoiesis. The marrow expansion can be linked to the high levels of erythropoietin found in these patients (Modell 1984). In β-thalassemia, the bone marrow expansion is increased relative to the extent of anemia because, Hb F, which is increased in these patients, has an increased affinity for oxygen in comparison to Hb A. This increased affinity means that, at a given oxygen saturation, Hb F gives less oxygen to the tissues than the same amount of Hb A, making adaptation to the anemia difficult (Modell 1984).

Patients with homozygous β-thalassemia major present during the first year of life, as γ-globin production decreases. The typical laboratory picture of a non-transfused β-thalassemia patient is severe hypochromia and microcytosis, marked anisocytosis and poikilocytosis with teardrop-shaped red cells, polychromasia, and basophilic stippling (Bunn 1986). There is a high level of nRBC. Evidence of hemolysis, including increased levels of unconjugated bilirubin is also observed (Bunn 1986). The microcytic red cells are caused by the cells undergoing an extra division, due to the negative regulation on cell division by hemoglobin content. Due to the high level of ineffective erythropoiesis, reticulocyte counts are not markedly elevated (Modell 1984).

If untreated, congestive heart failure usually leads to death within five years (Piomelli 1995). Hepatosplenomegaly also becomes an increasing problem. Along with the splenomegaly, secondary hypersplenism leading to thrombocytopenia, leukopenia, and rapid destruction of transfused red blood cells can develop.

In a study of Italian patients born after 1960, Borgna-Pignatti observed that heart disease was the most frequent cause of death, being directly responsible for 71% of the deaths. Infections (12%) and liver disease (6%) were the second and third most frequent causes of death, respectively. Survival to the age of 15 (highest age in the youngest cohort) increased with more recent births. Ninety-five (95% CI: 92-97)% of patients born from 1970-74 were alive at the age
of 15, compared to 97 (95% CI: 94-98)% of those born from 1975-79, and 98 (95% CI: 93-100)% of those born from 1980-84 (Borgna-Pignatti 1998).

1.1.3.1. Delay in Linear Growth
Around nine to ten years of age, patients with β-thalassemia may exhibit significant and noticeable retardation in linear growth (Bunn 1986), which is often more than one standard deviation below normal for age (Logothetis 1972). Growth failure before the age of four or five is generally attributed to folate deficiency or in association with a grossly overactive marrow and can usually be corrected by folic acid therapy, initiation of or increase in transfusion scheme, or splenectomy (Modell 1984). Although hemoglobin concentration does not appear to be directly correlated to growth, various systemic abnormalities that generally reflect the severity of illness (i.e., hepatosplenomegaly, cephalofacial bone deformities and cutaneous siderosis) showed a tendency to parallel increases in growth retardation (Logothetis 1972). Thalassemic children also exhibit a lack of muscular tissue, reflected in low 24-hour urinary creatinine. Urinary creatinine accurately reflects amount of muscular tissue as the only source of urinary creatinine is from the breakdown of muscle creatinine, which occurs at a rate of 2.0% per day (Modell 1984).

1.1.4. β-Thalassemia Intermedia
Thalassemia intermedia refers to patients who have β-thalassemia mutations but do not require regular transfusions. Most of these patients present with anemia later in life than transfusion-dependent β-thalassemia major patients (Wainscoat 1987). Thalassemia intermedia encompasses a wide spectrum of clinical disease; from adults with minimal symptoms to children just surviving without transfusions. The β-thalassemia intermedia phenotype is generally attributed to three main molecular causes: homozygous β-thalassemia with an ameliorating factor; δβ-thalassemia or Hb Lepore; or, heterozygous β-thalassemia with a worsening factor.

Patients who are homozygous for β-thalassemia may show an intermedia phenotype if they have β+ mutations. These are mutations, including those in the promoter region of the β-globin gene and a T to C substitution at IVS #1-6, that reduce, but do not eliminate the production of β-globin. Since β-globin production is not eliminated, there is less globin chain imbalance, leading to a less severe disease (Wainscoat 1987). The co-inheritance of α-thalassemia will also
reduce the globin chain imbalance, and severity of disease by decreasing the production of α-chains. This is more pronounced in patients with β⁺-thalassemia than in those with β⁰-thalassemia. While a single 3.7-deletion (-α/αα) is usually enough to produce a thalassemia intermedia phenotype in a patient homozygous for β⁻ mutations, two 3.7-deletions (-α/-α) or non-deletional silencing of α₂ is found if a patient homozygous for β⁰ mutations achieves a transfusion-independent state (Cao 1994). The third group of homozygous β-thalassemia patients who remain transfusion-independent are those with enhanced γ-globin production. Once again, this is due to the fact that globin chain imbalance is reduced. Enhancement of γ-globin production can be caused by the co-inheritance of an HPFH mutation, which may or may not be linked to the β-globin gene cluster or by a haplotype associated with high Hb F, including Xmn 1 site polymorphism in which there is a C to T substitution at -158 to the Gy-globin. Some of these associated haplotypes (such as the Xmn 1 site polymorphism) appear to only express high levels of Hb F in the face of erythropoietic stress (i.e., thalassemia), while others (such as single base substitutions at -117 and -196) are also expressed in non-thalassemic people. Most patients with homozygous or doubly heterozygous β-thalassemia, who display β-thalassemia intermedia phenotypes, have one or two mild β-thalassemia alleles (i.e., β⁺). (Camaschella 1995, Ho 1998, Rund 1997).

δβ-thalassemia patients produce neither δ- nor β- globin, with homozygotes showing 100% Hb F (Wainscoat 1987). The δβ deletion brings remote promoter sequences into the vicinity of the γ-globin gene, which may lead to enhanced production (Cao 1994). Hb Lepore, caused by a non-homologous crossing over between the δ and β genes can, in the homozygous state, produce thalassemia intermedia, through an increase in Hb F.

Lastly, patients with thalassemia trait (heterozygous for a β-globin mutation) can exhibit thalassemia intermedia phenotypes if there is a co-inheritance of extra α-globin genes. This acts in an opposite manner from the co-inheritance of β- and α-thalassemia; the triplicated, or quadrupled α-globin genes increase the globin chain imbalance. Co-inheritance of thalassemia trait and syndromes of inclusion body formation or hemolysis is also a proposed mechanism for the observation of β-thalassemia intermedia (Wainscoat 1987).
Ho examined 86 patients with β-thalassemia intermedia followed in the U.K. Sixty-five of the patients were homozygous or doubly heterozygous for β-thalassemia mutations, while 22 had a single β-thalassemia allele (Ho 1998).

The distinction between thalassemia intermedia and thalassemia major is unclear, but is generally made based on the following criteria: (1) age and hemoglobin concentration at presentation; (2) identification of one or two β* allele(s); (3) identification of co-inherited α-thalassemia (Wasi 1985).

1.1.5. Hb E/β-Thalassemia

Hemoglobin E, (α2βE2), is caused by a single substitution in the β-globin gene. A G to A substitution at codon 26 yields lysine instead of glutamic acid (Orkin 1982) creating a cryptic splice site; thus, less mRNA is transcribed and less hemoglobin is ultimately produced (Weatherall 1998). In addition, the substituted amino acid occurs at the α1/β2 contact site in the translated protein, reducing the affinity of the β-chain for the α-chain (Lachant 1987). The net negative charge of the βE-globin chain is less than that of the β-globin chain, reducing its attraction to α-globin, which has a net positive charge. The importance of the location of the mutation is highlighted by the fact that hemoglobins C and Oαβ have the same mutation (GLU to LYS), at different locations (codon 6 and 121, respectively), but remain oxidatively stable.

Hb E, in itself does not cause clinical problems. Patients hetero- or homozygous for Hb E follow a clinically benign course (Weatherall 1998). Furthermore, Poottrakul reported normal apoptosis and cell survival in a patient with homozygous Hb E (Poottrakul 2000) and the overall deficit in globin synthesis in homozygous Hb E is about equivalent to that seen in thalassemia trait (Bunn 1986).

Double heterozygosity for Hb E and a β-thalassemia mutation leads to Hb E/β-thalassemia, which shows a phenotype ranging from β-thalassemia intermedia to β-thalassemia major. One proposal for the unexpected severity of some cases of Hb E/β-thalassemia is that the excess of α-globin chains in Hb E/β-thalassemia might cause enough oxidative stress to accelerate the denaturation and precipitation of Hb E, which is oxidatively unstable. This might cause increased hemolysis, leading to a more severe disease (Bunn 1986).
A large variability is seen in the anemia of patients with Hb E/β-thalassemia. A report of 802 patients in Thailand showed a range of steady-state hemoglobin concentrations from 2.6 to 13.3 g/dL, with a mean of 7.7 g/dL (Fucharoen 1984). The level of anemia did not appear to be related to the β-thalassemia mutations or the Hb E genes. All patients had β^0-thalassemia mutations and it had been shown that Hb E levels do not differ greatly between people with different levels of anemia. Xmn 1 site status may explain some of the variations seen in hemoglobin concentrations. Winichagoon reported 90 patients, who were stratified into three groups, based on Xmn 1 site status. The eight patients who were +/+ had significantly higher total hemoglobin and Hb F concentrations than the 64 patients who were +/- and the 18 who were -/- (Winichagoon 1993). However, when Rees examined 25 patients in the U.K. and 16 patients in the U.S., he found no correlation between Xmn 1 site status and hemoglobin concentration (Rees 1998).

The disease is most prevalent in and around Thailand, with Indonesia, Bangladesh, Northeast India, and Sri Lanka also being heavily affected (Rees 1998). It is hypothesized that this mutation confers survival advantage by increasing resistance to malaria. Individuals with Hb E/β-thalassemia have been shown to be resistant to inoculated *P.vivax*, but not inoculated *P.falciparum* (Lachant 1987).

It is predicted that, in Sri Lanka, more than 2000 patients will require treatment for thalassemia at any given time, which would use approximately 5% of the annual expenditure on health (de Silva 2000). About 40% of these patients would have Hb E/β-thalassemia. Thus, Hb E/β-thalassemia represents a significant health burden.
1.1.6. The Spleen
The spleen, which is the largest single mass of lymphoid tissue in the body, lies in the upper quadrant of the abdomen and has both immunological and hematopoietic functions.

1.1.6.1. Normal Anatomy and Physiology
The spleen has an average weight of 125 g in adults, and an approximate length of 10.7 cm (Pearson 1995).

Surrounding the spleen is a capsule of dense connective tissue from which fibrous trabeculae extend into the splenic parenchyma (Ross 1995), which is composed of two distinct tissue types: red pulp and white pulp (Pearson 1995, Ross 1995).

The white pulp is composed of lymphatic tissue, containing a large portion (20 - 30%) of the total body lymphocyte mass (Crosby 1963). Other cells found in the white pulp include macrophages, plasma cells, and dendritic reticulum cells. The periarterial lymphatic sheath surrounds the splenic artery (termed the central artery in the white pulp) and is composed of T lymphocytes, plasma cells, and activated lymphoid cells and macrophages (Pearson 1995, Ross 1995). In general, the B-lymphocytes form the nodules and are surrounded by T lymphocytes. The germinal centers that are found in the nodules are reactive centers that form in response to antigen exposure. The enlarged nodules are termed splenic nodules or Malpighian corpuscles (Eichner 1979, Ross 1995).

The red pulp, the largest component of the spleen, is so called due the large number of red cells that are within its cords and sinuses, giving it a macroscopically red appearance. The red pulp is composed of splenic sinuses separated by splenic cords (termed cords of biliroth). These splenic cords are a loose meshwork of reticular cells fibbers that contain large numbers of erythrocytes, macrophages, lymphocytes, plasma cells, and granulocytes (Ross 1995). The macrophages within the splenic cords are responsible for the phagocytosis of damaged red blood cells, which begins the process of hemoglobin breakdown and iron reclamation. Other functions carried out by the red pulp include blood filtration, cytokine production, immune regulation, fetal hematopoiesis, regulation of blood volume, and a blood reservoir. It is the primary site of reticuloendothelial function in the spleen (Eichner 1979).
1.1.6.1. Splenic Circulation
Approximately 300 mL/minute of blood flows through the adult spleen, representing 5-6% of the total body blood volume (Eichner 1979, Pearson 1995).

The splenic artery, the largest branch of the celiac trunk, is the arterial blood supply of the spleen. The splenic artery divides into five branches before entering the spleen at the hilum, at which point it continues to divide into increasingly smaller blood vessels that enter the white pulp at the trabeculae. The central artery then sends branches into the white pulp itself, and to the sinuses located along its perimeter called marginal sinuses. When the central artery reaches the red pulp, it branches into arterioles called penicillin or penicillar arterioles (Ross 1995). The penecilli continue as arterial capillaries, which may be surrounded by aggregations of macrophages. Although it is agreed that the blood cells leave the vascular system to populate the red and white pulp, and then reenter the vascular system in the red pulp, the manner in which this is accomplished is debated. Both an opened and a closed system have been proposed, with the former being the currently accepted model (Eichner 1979, Ross 1995).

The open circulation hypothesis proposes that the splenic arterioles empty directly into the reticular meshwork of cords, rather than connecting to the endothelium-lined splenic sinuses. The blood then reenters the circulation by entering a sinus from the extravascular side after percolating through the cords and being exposed to the macrophages of the cords. Experimental evidence shows that red blood cells travel through the endothelium of the sinus, presumably reentering the vascular system from the red pulp cords. The blood collected in the sinuses drains to the trabecular veins that converge into larger veins and eventually the blood leaves the spleen when several tributaries meet at the hilum and emerge from the spleen as the splenic vein (Eichner 1979, Pearson 1995, Ross 1995).

1.1.6.1.2. Functions of the Spleen
The spleen has two main functions; in immunity as part of the lymphocyte system; and in storage of red blood cells and filtering of blood.

The white pulp is the location of immune functions, which include proliferation of lymphocytes, differentiation of effector lymphocytes and plasma cells, and secretion of humoral antibodies.

The hematopoietic functions of the spleen include hematopoiesis during fetal life, recycling of iron from red cell hemoglobin, storage of blood, and filtration of blood. Due to the large blood
volume received by the spleen, it is able to remove and destroy senescent, damaged and/or abnormal red blood cells and platelets (Eichner 1979, Pearson 1995, Ross 1995). Macrophages embedded in the reticular meshwork of the red pulp are responsible for removing these cells to be broken down by lysosomes. (Pearson 1995, Ross 1995).

In the spleen, the red cells follow a tortuous route where they are exposed to periods of stasis and come into intimate contact with splenic macrophages. This prolonged stasis exposes the red cells to hemoconcentration, hypoglycemia, and low pH; which may allow the spleen to filter out minimally damaged cells. In these minimally damaged cells, the prolonged stasis leads to further damage of the cell membrane, and if the damage is of sufficient degree, phagocytosis follows (Eichner 1979). This process, termed culling, occurs in the center of the cord. The ability of the macrophage to recognize old or abnormal red blood cells is not well understood. It is hypothesized that red cells become more rigid as they age and are thus more easily trapped in the meshes of the red pulp (Ross 1995). A second suggested mechanism involves the immune system, which may respond to changes in the surface of the erythrocytes, and tag pathologically modified cells with opsonizing antibody. As evidenced in patients who have undergone splenectomy, the bone marrow and liver also participate in the filtering function of the red blood cells (Pearson 1995).

The spleen has the ability to remove intra-erythorcytic particles or inclusions from intact red blood cells without destroying the red cells. This process, termed pitting, removes such particles as siderotic granules, Heinz bodies, Howell-Jolly bodies, red cell nuclei, malarial parasites, Bartonella organisms, and autophagic vacuoles (Pearson 1995). The damaged cells must pass through minute apertures with pores of 0.5 to 2.5 μm between the endothelial cells of the splenic cords and their fenestrated basement membranes, as they move from the endothelium of the splenic cords to the sinus (Ross 1995). The deformability of normal red blood cells allows them to easily make this passage. However, non-compressible inclusions, such as Heinz bodies, obstruct this passage. A small projection of the cytoplasm, containing the intracellular particle, lags behind and the membrane is stretched around it, and pinches of the small section, which is phagocytized. The remaining red cell, now free of debris, has the deformability of a normal red blood cell and can continue to circulate for four months. After this time, the cells lose enzymatic activity and membrane plasticity and are trapped and destroyed by the spleen (Crosby 1963).
The delayed microcirculation in the spleen allows time for splenic phagocytes to remove even poorly opsonized bacteria (Pearson 1995). It also facilitates the mounting of an immune response to intravenously administered particulate agents. As the blood enters the spleen, the soluble antigens are skimmed off with much of the plasma to enter the right-angled arterioles supplying the germinal centers of the white pulp, but particulate antigens lodge first in the red pulp. Within hours these are transported, possibly by mobile macrophages, across the marginal zone into the germinal center where immunoglobulin M (IgM) antibody response is initiated (Pearson 1996, Ross 1995).

1.1.6.2. Splenic Pathophysiology in β-Thalassemia

Splenomegaly is a consequence of both extramedullary erythropoiesis, and a consequence of the abnormal red cells, which are constantly presented to the spleen (Modell 1984). The spleen forces red blood cells to pass through narrow vessels, which requires great flexibility. The thalassemic cells, due to altered membrane and inclusion bodies, are not able to easily pass through these vessels. These cells (or parts of them) become trapped, thereby impeding circulation. Thus, the spleen becomes engorged and enlarged. This reduced circulation in the spleen damages all blood cell types, to different degrees. Thalassemic red cells, by virtue of their rigidity are destroyed first, then transfused red blood cells, leukocytes, and lastly platelets. Splenomegaly also increases intravascular blood volume and can lead to red cell sequestration in the large third space (Bunn 1986).

Hypersplenism generally describes a syndrome with the following symptoms: (1) splenomegalgy; (2) a reduction in one or more cellular elements of blood; (3) compensatory bone marrow hyperplasia; and (4) correction of cytopenia following splenectomy (Crosby 1963). Even in transfused patients, despite the absence of severe splenomegaly, hypersplenism, to various degrees is generally seen by the age of eight or nine years (Piomelli 1995). Hypersplenism leads to an increase in plasma volume and an increase in the intrasplenic pool of red cell in the pulp cords. This latter effect is not always in proportion to spleen size, so that spleen measurement may give an inaccurate reflection of this effect (Nightingale 1972).

1.2. Therapy of β-Thalassemia

The mainstay of therapy in β-thalassemia is regular red cell transfusions. Newer, non-transfusion based approaches are being attempted.
1.2.1. Regular Red Cell Transfusions

Although initial reports reported that transfusions did not help the clinical picture of disease (Whipple 1936), regular red cell transfusions have become the mainstay of treatment (Rund 2000). Orsini first introduced regular red cell transfusions in 1961; previously patients had been transfused only when symptomatic (Piomelli 1995). In 1969, Piomelli reported four patients maintained with transfusions to hemoglobin concentrations between 10.5 and 11.9 g/dL. He reported that all four patients exhibited normal heart size, growth at or above the 25th percentile, no bone changes and no evidence of thal facies. In addition, three of the patients had livers and spleens of less than three centimeters (Piomelli 1969). Regular red cell transfusions suppress endogenous erythropoiesis, preventing hypertrophy of ineffective bone marrow. Growth and development may be normal (Weatherall 1997).

There are three main issues in transfusion therapy: (1) minimize iron overload by transfusing to the minimum hemoglobin needed to assure growth and a good quality of life; (2) ensure blood is free of blood-borne viral agents; and (3) reduce the incidence of transfusion reactions and minimize immuno-suppression reactions by using leukocyte filters (Rund 2000). The current practice in most centers is to maintain a baseline hemoglobin concentration of 9.0 – 10.0 g/dL. This level achieves the goal of suppressing endogenous erythropoiesis while minimizing the amount of iron overload induced (Cazzola 1995, Piomelli 1995, Rund 2000) by regular red cell transfusions, which causes damage to the heart, liver, pancreas, endocrine, and other organs. It is the major cause of death from β-thalassemia in the second decade of life (Bunn 1986).

Other than iron-overload, the major problems with transfusions remain the assurance of sufficient quantity and quality of blood, avoidance of adverse transfusion reactions, including alloimmunisation and transmission of viral infections, and the expense (Politis 1989).

1.2.1.1. Iron Chelation

Every milliliter of transfused red blood cells is assumed to add 1 mg of iron to the human body. Because humans are designed to conserve as much iron as possible and do not possess an adequate system for the removal of this excess iron; patients on regular red cell transfusions require iron chelation therapy (Piomelli 1993). Furthermore, iron overload has also been documented in thalassemia intermedia patients, who do not receive regular transfusions. This may be attributed, in part, to the increased number of early red cells in the bone marrow of these patients, due to maturation arrest (Modell 1984). Since early red cells have more TfR, the
marrow may be more avid for iron. The liver and heart are major repositories for the transfused iron, with myocardial disease being the life-limiting complication of transfusional iron overload (Olivieri 1997).

Body iron burden is accurately determined by the analysis of liver iron concentration by liver biopsy and chemical analysis. Although normal liver iron concentration is between 0.6 and 1.6 mg iron/gm liver, dry weight, the ideal range for transfused patients is between 3.2 and 7.0 mg iron/gm liver, dry weight. This range makes a compromise between the toxicity of increased iron burden and toxicities from desferoxamine administration. Liver iron concentrations above 7.0 mg iron/gm liver, dry weight are correlated with increased risk of hepatic fibrosis, diabetes mellitus, and other complications. Liver iron concentrations greater than 15.0 mg iron/gm liver, dry weight increased risk of cardiac complications and early death (Olivieri 1997). The current gold standard for the chelation of transfusional iron is nightly subcutaneous administration of desferoxamine.

1.2.2. Bone Marrow Transplantion (BMT)

BMT provides a curative treatment for β-thalassemia. The first successful report of BMT for thalassemia was performed in 1981 (Thomas 1982), after which the patient remained disease free for more than 11 years post-transplant (Walters 1994). Pesaro has the largest experience in BMT to date, and they have reported an event-free survival rate of 72% in the 826 who have undergone BMT (Lucarelli 1998). The overall survival in these patients was 78%. Analysis of 161 patients, who had undergone BMT prior to September 1989, identified three risk factors that adversely affected both survival and event-free survival. These three factors were: (1) liver greater than 2 cm below costal margin, (2) presence of portal fibrosis, and (3) an irregular chelation program. On the basis of these factors, the Lucarelli Classification was created. Patients in class 1 had none of these risk factors, patients in class 2 had one or two of these risk factors, and patients in class 3 had all three risk factors. Survival and event-free survival were 97% and 94% respectively in class 1, 84% and 81% in class 2, and 54% and 49% in class 3 (Lucarelli 1990). Long-term complications including fulminant sepsis, growth impairment, and graft-versus-host disease have been reported following BMT in patients with β-thalassemia (De Simone 1995, Piga 1998). Other possible long-term affects of BMT include infertility and secondary tumors (Apperley 1993).
1.2.3. Gene Therapy

Gene therapy in β-thalassemia covers three general strategies: (1) the addition of an exogenous gene to human hematopoietic cells by gene transfer; (2) the correction of mutation or the mutant RNA transcript through the use of molecular biological 'tricks'; or (3) the down regulation of the α-globin gene to reduce the chain imbalance (Rund 2000).

Some obstacles in gene transfer therapy for β-thalassemia include the need for high levels of precise, cognate, tissue specific regulation of β-globin production so that it balances with the α-globin production; the need to introduce the entire β-globin gene, as opposed to a smaller section, due to the heterogeneity seen in mutations causing β-thalassemia; and the possible need to eradicate some or all of the genetically defective residual hematopoietic stem cells (Gale 1989).

One biological trick is the introduction of oligonucleotides targeted to specific regions in the DNA double helix which contain mutations, exploiting the cell's own repair mechanism (Wang 1996).

The use of methods to downregulate α-globin gene expression is most useful in patients with β-thalassemia intermedia who have a higher level of β-globin gene output (Rund 2000).

One novel approach in gene therapy for β-thalassemia could be the insertion of an EPO-vector with an adeno-associated virus-mediated gene transfer for patients who had shown unambiguous responsiveness to a short-term trial of rhEPO (Bohl 2000).

1.2.4. Pharmaceutical Augmentation of Fetal Hemoglobin

Patients with HPFH produce neither Hb A nor Hb A₂, yet they remain clinically well into adulthood (Forget 1998). If the γ-globin gene in patients with β-thalassemia could be re-activated, β-thalassemia patients could theoretically obtain an HPFH phenotype.

It has be shown that genes in which the cytosine residues are methylated at CpG sequences are not expressed, while those that are not methylated at CpG sequences can be expressed. In adults, the γ-globin gene is highly methylated, while the γ-globin gene remains hypomethylated in fetuses. Furthermore, van der Ploeg and Flavell have shown that there is a negative correlation between the methylation status of the β-globin locus and its activity; the human γ-
globin genes are unmethylated in tissues where they are expressed, but heavily methylated in tissue where they are not expressed (Ley 1991, van der Ploeg 1980). Thus, hypomethylation appears to be correlated with expression of the γ-globin gene (Alter 1988).

The suppression of transcription by the methylation of DNA has been shown through mouse retrovirus experiments (Bird 1984). In mouse cells, methylation of one or more CpG sequences between 760 bp upstream and 100 bp downstream of the 5' end of the γ-globin gene abolishes transcription, while methylation of other sequences did not abolish transcription (Bohl 2000). Fetal calf serum promotes Hb F production in cultures, while fetal sheep serum inhibits Hb F production (Constantoulakis 1990, Groudine 1986). Cells from a patient with β⁺-thalassemia were cultured in the presence of fetal calf serum or fetal sheep serum and methylation sensitive restriction enzyme sites were used to demonstrate the relative levels of methylation in the two culture types. No difference was seen in the methylation status of sites 5' to the δ-globin gene or 3' to the β-globin gene. However, cultures grown in fetal sheep serum exhibited a diminution of the hypersensitive sites 5' to the γ-globin genes (Groudine 1986).

Further evidence of the importance of methylation on the expression of the γ-globin gene was provided by showing that signal specific protein binding to the proximal γ-promoter, which is involved in the preferential interaction of the γ-promoter and the LCR, is inhibited by methylation (Jane 1993, van der Ploeg 1980). It was hypothesized that drugs that could induce the hypomethylation of the γ-globin, may augment Hb F synthesis in patients with β-thalassemia, alleviating the α/non α globin chain imbalance and reducing the morbidity of the disease.

Additionally, it has been shown that disturbances in erythroid kinetics can cause an unusually high proportion of cells to follow the high F pathway (Blau 1993, Stamatoyannopoulos 1985). Drugs that induce these states, including S-stage drugs that lead to significant cytoreduction of late progenitor pools, may thus be able to induce Hb F expression (Stamatoyannopoulos 1985). Subsequently, other drugs, including short chain fatty acids and hematopoietic growth factors have shown an ability to augment Hb F synthesis.
1.2.4.1. 5-Azacytidine (5-azaC)

5-azaC is an antineoplastic agent that acts by being incorporated into DNA in the place of normal cytosine residues during DNA synthesis, leading to a marked demethylation of the DNA (Pearson 1996). This demethylation is presumably caused by a reduction or cessation of the activity of DNA methyltransferase (Stamatoyannopoulos 1992).

The augmentation of Hb F was first attempted in anemic baboons, using 5-azaC (DeSimone 1982). DeSimone reported two low Hb F responders who had baseline Hb F levels of 2% and peak Hb F levels of 32% and 33%. He also reported two high Hb F responders who achieved peak Hb F levels of 67% and 81% from baselines of 10%. There was a corresponding decrease in Hb A levels, with an increase in \( \gamma/(\gamma + \beta) \) synthesis ratios to 0.40 and 0.39 in the low responders, and 0.71 and 0.85 in the high Hb F responders; initial ratios were not provided. The major effect of 5-azaC appeared to be an increase in Hb F per F-cell as opposed to an increase in the number of F-cells, as the increase in Hb F was proportionally greater than the increase observed in F-cells (DeSimone 1982).

The original hypothesis for the mechanism of action of 5-azaC was the demethylation of the \( \gamma \)-globin gene so that it resembled the fetal hypomethylated state and, initial animal studies did indicate a rapid and reversible methylation of DNA after treatment (Charache 1983, Ginder 1984). However, Humphries showed that this hypomethylation was present in a patient who failed to respond to treatment with 5-azaC (Humphries 1985), and others showed that the \( \varepsilon \)-globin gene, which was not expressed, was also hypomethylated during treatment (Wood 1983). Furthermore, there is some evidence that, during normal development, the \( \gamma \)-gene is rendered inactive before its methylation, thus questioning the causal link of methylation to globin gene expression (Enver 1988). It was thus proposed that 5-azaC worked, at least in part, by inducing an acute anemia like state. It was further shown that progenitors cultured in 5-azaC continue to produce Hb F for up to 12 days after being replated into drug-free media (van der Ploeg 1980). This would support that Hb F induction is through the recruitment of more primitive progenitors committed to a fetal program.

In the chicken, the \( \rho \)-globin gene acts in a similar manner to the \( \gamma \)-globin gene in humans; both are the predominant non-\( \alpha \)-globin in the fetal stage, but are essentially turned off in the adult stage (Ginder 1984). Sites in the 5' upstream putative control regions of the \( \rho \)-globin in the chicken are highly methylated in adult chicken reticulocytes, but completely unmethylated in 5-
day embryonic red cells. Treatment with 5-azaC resulted in demethylation around the \( \beta \)-globin gene. RNA isolated from reticulocytes of treated adult chickens showed small amounts of \( \beta \)-globin RNA, while RNA isolated from controls showed no \( \beta \)-globin RNA.

Most studies of 5-azaC in patients with \( \beta \)-thalassemia have shown little or no response (Pearson 1996). In 1982, Ley reported the use of 5-azaC in a patient with \( \beta \)-thalassemia. The \( \alpha / \) non-\( \alpha \) globin chain synthesis ratio decreased from 2.9:1 at baseline to 1:1 during therapy, and 2:1 two weeks after the five days of therapy stopped. Percent Hb F increased from 1.6% at baseline to a peak of 20.8% at day 40. An increase in total hemoglobin concentration was also reported. However, the patient received two transfusions during the five days of therapy (145 mL on day two and 165 mL on day five) to maintain hemoglobin concentration above 8.0 g/dL. The hemoglobin concentration did not fall to below this level again during the 40 days of follow-up (Ley 1982). A report of four patients reported an increase in total hemoglobin from 5 g/dL to a peak of 9 g/dL in one patient and Lowrey reported similar results in two patients treated for 12 months (Dunbar 1989, Lowrey 1991). Stamatoyannopoulos and Nienhuis reported an increase in total hemoglobin concentration from 3.5 g/dL to 7.0 g/dL in a patient with \( \beta \)-thalassemia (Stamatoyannopoulos 1992) indicating that the use of 5-azacytidine in patients with severe \( \beta \)-thalassemia is possible, although response may be unpredictable. Charache reported an increase in total hemoglobin concentration from 7.5 - 8.5 g/dL at baseline to 10 - 12 g/dL during treatment in a severely affect patient with sickle cell disease (Charache 1983). Increases in Hb F synthesis, \( \gamma / (\gamma + \beta) \) globin chain synthesis ratios, and red cell survival were also reported for the same patient.

Recently, Koshy treated eight adults with sickle cell anemia with varying doses of 5-AzaCdR (an analog of 5-AzaC), which also inhibits DNA methyltransferase causing DNA demethylation. An increase in total hemoglobin of at least 1.0 g/dL was seen in six patients. An increase in Hb F from 3.5 \( \pm \) 2.5 (0.5 - 8.3)% at baseline to 13.5 \( \pm \) 3.7 (8.6 - 20.2)% was attributed to both an increase in the number of F-cells and Hb F per F-cell. \( \gamma / \) non-\( \alpha \) globin chain synthesis ratios increased from 3.2 \( \pm \) 1.4 (<1.0 - 5.0)% at baseline to 13.7 \( \pm \) 4.3 (6.4 - 22.0)% which is a significant increase (Koshy 2000). Five of the patients who responded were patients who had previously been unresponsive to therapy with hydroxyurea. Six patients were treated with at least two different doses of 5-AzaCdR and, within a given patient, increasing the dose of 5-AzaCdR led to a higher maximum increase in Hb F.
1.2.4.2. Other S-phase specific agents
Cytosine arabinoside (AraC) given to anemic baboons led to an increase of F-reticulocytes as a percentage of total reticulocytes and an increase in $\gamma/\beta$ globin chain synthesis ratios (Papayannopoulou 1984). During, or immediately after treatment, there was a significant reduction in the size of late erythroid progenitor pools (i.e., CFU-e and e-clusters), followed by a drastic increase. There was no significant effect on the pool of BFU-e, supporting the hypothesis that S-stage specific agents act on cells undergoing cycling (Papayannopoulou 1984). F-cell formation may be stimulated as a result of increasing the chances of premature terminal commitment of immature cells that have an active Hb F program (Stamatoyannopoulos 1992).

Bone marrow cells incubated with AraC synthesized excess $\gamma$-globin, as evidenced by increased proportions of Hb F-positive erythroblasts, CFU-e colonies, and e-clusters (Galanello 1988, Stamatoyannopoulos 1992). However, DeSimone reported only a slight increase in Hb F levels with the treatment of anemic baboons with AraC (Al-Khatti 1988, DeSimone 1982).

AraC, along with other S-phase specific drugs such as vinblastine, methotrexate, and busulfan are capable of augmenting Hb F without inhibiting DNA methylation. However, in order to maintain increased levels of Hb F, chronic administration at toxic doses is necessary. Since the carcinogenic effects remain unknown, the search for less toxic agents, requiring a less rigorous administration schedule was undertaken.

1.2.4.3. Erythropoietin (EPO)
EPO is a 35 kD glycoprotein hormone secreted by the kidney that induces the formation of red blood cells by stimulating proliferation, differentiation and maturation of the cells and has been shown to increase Hb F in bone marrow cultures (Papayannopoulou 1976).

Recombinant human EPO (rhEPO) has been used to treat the anemia of patients with end stage kidney disease, several of whom also had either $\beta$- or $\alpha$-thalassemia trait. These patients needed higher than normal doses of rhEPO to increase hemoglobin concentration, but these concentrations became higher than is generally seen in thalassemia trait patients (Rachmilewitz 1998). Anemic baboons and experimental thalassemic mice showed good responses to rhEPO,
if it was used at doses that were five to ten times those used to treat dialysis patients (Al-Khatti 1987, Leroy-Viard 1991).

Trials in patients however, have shown mixed results. Two short-term trials showed a mean increase of 2 to 3 g/dL of total hemoglobin, while a third reported no increase in total hemoglobin concentration in the six patients it studied (Olivieri 1992, Rachmilewitz 1991, Sher 1994). Only one patient reported an increase in Hb F levels. A longer trial of rhEPO for patients with thalassemia intermedia indicated a dose-dependent increase in total hemoglobin concentration of 2 to 3 g/dL (Aker 1994). In this trial, splenectomized patients were reported to have had a better response than the non-splenectomized patients have. Rachmilewitz (1998) hypothesized that this was because the newly formed thalassemic red blood cells had a better chance of survival if there was no intact enlarged spleen. Accelerated linear growth was seen in the pediatric patients and no major side effects were seen during the long-term administration of rhEPO. Olivieri (1995) reported a patient who has shown a sustained response to rhEPO. No correlation between response and endogenous EPO levels were observed in any of the studies.

Bohl et al. used an adeno-associated virus-mediated gene transfer of mouse EPO cDNA to deliver EPO to β-thalassemic mouse for up to 12 months. All mice showed an increase in total Hb concentration and improvement was correlated to serum EPO concentration ($r^2=0.749; P=0.026$). Six of eight mice showed an improvement in non α/α globin chain synthesis ratio, although none achieved a ratio of 1.0. There was a 2- to 3-fold decrease in α-globin precipitates found in erythrocyte ghosts in the treated mice, although there was still much more than seen in non-thalassemic mice. The improvement in excess α-globin chain production correlated to hemoglobin improvement, $r^2=0.716$ ($P=0.034$). The effective erythropoiesis observed was maintained for at least 12 months (Bohl 2000). BFU-e, which are programmed for β-globin synthesis, may be preferentially mobilized by the EPO. This, together with the suppression of expansion by late erythroid progenitors, increased effective erythropoiesis, while not aggravating ineffective erythropoiesis. Previous studies, using different, less effective gene transfer methods, showed only partial and inconsistent responses, much the same as that seen in human studies. It is therefore likely that the chronic expansion of the BFU-e compartment, necessary for a sustained response, requires intense and sustained stimulation to be effective (Bohl 2000).
1.2.4.4. Sodium Phenylbutyrate (SPB)

It was observed that infants of diabetic mothers have higher levels of Hb F than infants born to non-diabetic mothers. Bard compared 9 infants of diabetic mothers to 9 control infants; all of the infants of diabetic mothers synthesized levels of Hb F above the 95% confidence interval for gestation age, while only 1 of the control infants was outside of this range (P<0.001) (Bard 1985). In addition, Hb F was higher in the infants of diabetic mothers 91.2 ± 2.7% vs. 87.9 ± 3.1% (P<0.05). Perrine also noted that infants of diabetic mothers had significantly lower synthesis of β-globin than did other infants (Perrine 1985). When plasma from the infants of diabetic mothers was added to in vitro cultures from normal infants, the usual peak in β-globin production, which occurs between days seven and 14, was stopped. Perrine thus hypothesized that something in the plasma of infants of diabetic mothers inhibits the switch from γ-globin production to β-globin production. The possibilities included insulin, IGF-1, isoleucine, and α-amino-n-butyric acid, all of which are high in infants of diabetic mothers.

1.2.4.4.1. Mechanisms of Action

Butyrates appear to act independently from any cytotoxic effects, by acting indirectly and relatively specifically on the γ-globin chain promoter to increase transcription. SPB is known to inhibit histone deacetylase, leading to acetylation of histones and alteration of chromatin structure (Riggs 1977). This may destabilize the nucleosome structure on transcriptionally active genes, leading to higher expression (Stamatoyannopoulos 1992). However, this is likely not the entire mechanism of action because the effects of butyrates are fetal-gene specific and are dependent on selected promoter elements in individually transfected genes (Ginder 1984, Glauber 1991).

It has been shown that only the proximal 61 base pairs of the γ-globin promoter are necessary for the upregulation of expression by butyrates, although the inclusion of up to −160 base pairs from the start site augments this upregulation (Faller 1995, Ikuta 1998). Ikuta (1998) performed in vivo footprinting in the sickle cell anemia patient and one of the β-thalassemia responders in his 1998 study. Four promoter regions were newly footprinted to different extents. These regions were named butyrate response elements G1 - G4 (BRE-G1 to BRE-G4). Several mutations that lead to a HPFH phenotype are in close proximity to the BRE sequences. It is possible that during therapy with butyrate, new nuclear protein binding to multiple regulatory sites of the γ-globin gene promoter developed. The erythroblasts respond by increasing γ-globin
mRNA and thus γ-globin protein. Further study of BRE-G1 was undertaken with EMSAs. BRE-G1 (-68 to -43 base pairs from start) was focused on because it falls within the region of 60 base pairs proximal to the promoter that is adequate to respond to upregulation by butyrates. In addition, the stage selector element, which is implicated in competitively silencing the β-globin gene in the fetal stage, is in this region. Prior to treatment, two weak DNA-binding activity sites to the BRE-G1 were seen. This was similar to the pattern seen in a normal control that hadn't been treated with butyrate. During and after treatment, three new strong DNA-protein complexes were generated with the patient's nuclear extracts. One of the new bands migrated in parallel with a protein-DNA complex detected in K562 cell nuclear extracts, while the other 2 migrated more slowly than the similar bands detected in K562 nuclear extracts. These two bands were not detected in nuclear extracts from non-erythroid cells (Raji and HeLa), even when these cell lines were treated with butyrates. Therefore, these two complexes are likely formed from nuclear factors that are erythroid-specific, while the first complex is likely formed by ubiquitous factor(s). Reporter gene assays with BRE-G1 were undertaken with a 335-base pair fragment of the γ-globin gene promoter and human growth hormone gene as a reporter. This fragment of the γ-globin gene promoter was previously shown necessary and sufficient for induction of γ-globin production by butyrates. Human growth hormone expression was compared between wild-type promoter and a promoter with mutations abolishing DNA-protein complex formation to BRE-G1 in EMSAs. In the wild-type construct, expression was enhanced six-fold over baseline following treatment with 1 mmol/L of sodium butyrate. No increase was seen when comparing treatment and no treatment in the mutant construct (Ikuta 1998). It seems likely, therefore, that butyrates act by increasing transcription of the γ-globin gene and that the newly footprinted regions observed during treatment with butyrate are necessary for the upregulation of this protein.

1.2.4.4.2. In Vitro Studies
In in vitro studies, only α-amino-n-butyric acid, and a derivative, SPB, showed an ability to augment γ-globin synthesis (Perrine 1985). Several other in vitro studies were carried out to determine the ability of butyrates to increase γ-globin synthesis. Perrine showed a significant increase in γ-globin synthesis in 16 of 20 cultures treated with either α-amino-n-butyric acid or SPB. A significant increase in Hb F production was reported in 12 samples, with a mean increase of 11.8% per BFUe. In ten cultures examined, a significant increase in the percent of F-positive BFUe was seen in eight, with seven showing increases of greater than 20%. The two
cultures that did not show an increase were from patients with baseline levels greater than 70% (less than one year of age). Partial corrections of α/non α ratios were seen in samples from patients with β-thalassemia. The proportion of α to non α globin chain synthesis in the control samples were 1.8 to 5.7, while those of the treated group had 36% less α globin excess (Perrine 1989). In contrast to previous studies with 5-azaC and hydroxyurea etc, which work through cytotoxic effects, the in vitro studies involving SPB showed no evidence of cellular toxicity; the pH of the culture media was not affected, the number of colonies was similar in the presence and absence of drug, and complete hemoglobinisation was seen in the erythroid cultures (> 15 pg total hemoglobin per cell).

1.2.4.4.3. Animal Studies

Animal studies have also been used to show the effectiveness of butyrate derivatives. To test the effectiveness of SPB at preventing the switch from primarily γ-globin production to primarily β-globin production, Perrine compared the β-globin chain synthesis levels in four ovine fetuses infused with SPB to 12 control fetuses (three infused with saline). In the control fetuses, β-globin production began at approximately gestation age day 112; it had increased to 45% of non-α-globin at day 125, and was 80 - 100% by birth (day 140 - 145). Infusions of SPB began at day 119 to 122; two of the fetuses had 10 - 15% β-globin at infusion start, and 40-50% at birth; a third had 4% β-globin at infusion start and none at birth. Thus, all three fetuses exhibited some delay in the globin gene switch. In the fourth fetus, 35% β-globin at infusion start increased to 85% at birth; there was no inhibition of the switch. Infusions were stopped at birth in all cows, and the switch to β-globin synthesis was complete, or nearly complete (88 - 100%) within 10 days (Perrine 1988). Hb F production also appears to be stimulated by butyrates in both anemic and non-anemic primates (Constantoulakis 1989, Constantoulakis 1989, Stamatoyannopoulos 1992).

1.2.4.4.4. Human Studies

The initial patient studies with butyrate derivatives were conducted with arginine butyrate, which must be administered intravenously. Perrine treated six β-hemoglobinopathy patients with 500 mg/kg/day for seven days, followed by a dose increase to 1500 - 2500 mg/kg/day for a total of two to three weeks. An increase in F-reticulocytes was seen in all patients, but this increase was larger in those with lower pre-treatment levels, this was sustained for at least a month in all patients. Also seen in all patients was an increase in γ-globin synthesis (6 to 45% above
baseline levels; P<0.01); an increase in γ-globin mRNA (two- to six-fold increase over baseline); and an improvement in non α/α globin synthesis ratios (one thalassemia major patient achieved ratios typical of thalassemia intermedia and one thalassemia intermedia patient achieved ratios typical of thalassemia trait). In one patient (homozygous Hb Lepore), who received extended therapy for five additional weeks, total Hb increased from a baseline of 4.7 g/dL to a peak of 10.7 g/dL; in addition, non α/α ratios which were 0.6 at baseline, increased to normal (1.0) with continuous infusions, and during maintenance therapy, remained at 0.8. These effects fell to below baseline levels within 12 - 48 hours after therapy was stopped (Perrine 1993). Ikuta saw similar results during the treatment of seven patients with β-thalassemia treated with SPB. Six of the patients showed an increase in γmRNA/αmRNA ratios of 2.8- to 6.0-fold after one to six weeks of treatment. The mean increase in γ-globin mRNA production was 2-fold over baseline. An increase in non α/α globin synthesis ratio was reported in one patient; from 0.40 at baseline to 0.84 after three weeks of treatment (Ikuta 1998). In 1998, Sher reported on the first extended trial of arginine butyrate in five patients with β-thalassemia and five with sickle cell disease. Initial doses of 500 mg/kg/day were increased to 1500-2000 mg/kg/day for five to six days per week for nine to 13 weeks (mean: ten weeks). Response, defined as an increase in total hemoglobin concentration of at least 2.0 g/dL over baseline, was not seen in any patient (Sher 1995). A second extended study, by Atweh, also reported disappointing results. Six patients were treated with 166 - 666 mg/kg/day for 5 days/week for three to 72 weeks (mean: 15 weeks). Three patients showed a temporary response. In one responder, treated for 72 weeks, Hb F and total hemoglobin concentration returned to baseline levels after 45 weeks, despite continuation of treatment (Atweh 1999). Recently, Atweh reported on his experience with pulse-butyrate therapy for extended periods of time. Eleven patients were treated with 250 - 500 mg/kg/day, increased to 500 mg/kg/day for 4 days, followed by a ten to 24 day break. Patients were treated for seven to 112 weeks (mean of 30 weeks). Nine of the eleven patients showed a mean increase in Hb F from 7.2 to 21.0% (P=0.002), with a positive correlation between baseline and peak Hb F (r=0.87, P=0.002). All patients showed an increase in F-cells of 27.5 to 42.5% over baseline (P=0.00040) and again there was a positive correlation between baseline and peak values (r=0.93; p<0.0001). Seven of the patients showed an increase in total hemoglobin concentration. The mean hemoglobin concentration rose from 7.8 g/dL at baseline to a peak of 8.8 g/dL (P=0.006). All responses were maintained throughout the course of treatment (Atweh 1999).
Early trials of arginine butyrate showed promising results with early phase I-II trials achieving biochemical activity in 80 - 90% of patients (Faller 1995). The effective dose for induction of γ-globin synthesis appeared to be between 500 - 1500 mg/kg/day with effective maintenance dose of 2 to 4.5 g/kg/month, administered over three to four nights, one to two times per month. A drug-free break of at least 10 days between courses provided the best results (Ikuta 1998). However, arginine butyrate must be given intravenously due its short half-life and because of the fact that it is rapidly metabolized in first-pass hepatic clearance; high plasma levels cannot be reached (Faller 1995). An orally active butyrate compound would facilitate treatment.

SPB, an orally active butyric acid compound, has been used for years in patients with urea cycle disorders. The drug has been administered for over 100 patient years, with no serious adverse effects reported (Dover 1998). A report of 15 patients treated with SPB for urea cycle disorders showed that the mean percentage of F-cells was higher than those seen in normal subjects (Dover 1998).

Collins treated eleven β-thalassemia patients with SPB at 20 g/day for 41 to 460 days. Eight of the eleven patients were transfusion-independent. A response, defined as an increase in total hemoglobin concentration ≥ 1.0 g/dL, was observed in 4 patients (all transfusion-independent). An increase in F-reticulocytes was seen in all patients, including those who did not show an increase in total hemoglobin concentration. The magnitude of increase was not correlated to response in Hb F. As in the initial studies with arginine butyrate, the increase in F-reticulocytes was sustained for at least one month after therapy. Response was correlated to baseline serum EPO concentration and Hb F levels. A serum EPO concentration greater than 120 IU/L was correlated with response. Four of six patients with concentrations above 120 IU/L responded, while none of the 5 patients below this level (P<0.05). Four of five patients with baseline Hb F levels greater than 40% responded, while none of the 4 patients with baseline Hb F levels below 40% responded (P<0.05) (Collins 1995).

In a recent study, Reich et al. used oral isobutyramide to reduce transfusion requirements in eight patients with β-thalassemia major. Prior to study, transfusions were reduced to maintain a hemoglobin concentration of 8.5 g/dL; patients were transfused whenever levels fell below this. This transfusion protocol was maintained during the 8 months of treatment. Response, defined as a decrease in body iron burden (from transfused blood) of at least 20% was seen in two patients (Reich 2000). All patients had an increase in Hb F from 3.1 (1.9 - 4.8)% at baseline to
6.0 (3.8 – 8.7)% during treatment (P=0.0017). At both baseline and during therapy, the Hb F levels were significantly higher in the responders than the non-responders.

1.2.4.5. Hydroxyurea (HU)

Ribonucleotides are converted to deoxyribonucleotides by the enzyme ribonucleoside diphosphate reductase. HU, (CH4N2O2) which inhibits ribonucleoside diphosphate reductase, thus inhibits DNA synthesis (Dorr 1994, Young 1964).

HU appears to be well absorbed from the gastrointestinal tract, and reaches peak serum levels within one to two hours after administration (Dorr 1994). HU is degraded by urease, an enzyme found in intestinal bacteria. The elimination half-life is 3.5 to 4.5 hours, with the remaining portion excreted intact in the urine (Dorr 1994). Within 24 to 48 after oral administration, 30 - 60% of the parent drug is eliminated through renal excretion (Donehower 1992).

1.2.4.5.1. Mechanisms of Action

Platt et al. (Platt 1984) treated two patients with Hb SS with HU at 50 mg/kg/day for five days. These patients had an increase in F-retics within 24-72 hours after treatment start. If the only mechanism of action were recovery after myeloid suppression, the increase would not have been so rapid. Thus, it was proposed that HU had an additional mechanism of action. Platt suggested that Hb F production was enhanced in very mature erythroid progenitors, or even erythroid precursors, meaning that HU stimulates erythroid differentiation. Post-treatment, there was a measurable loss of methylation (about 25%) in the region 5' to the γ-globin gene in vivo. Platt attributed this to increased γ-globin gene expression in selected cell populations or a consequence of increased γ-globin gene expression, rather than as a cause of Hb F enhancement (Platt 1984). Furthermore, in vitro studies have shown that HU has no direct effect on DNA methylation (Nathan 1985). HU appears to act by recruiting more early red blood cell precursors into the high F pathway (Pearson 1996) either through direct reprogramming of the late erythroid precursors or by accelerating the turnover of erythroid precursors with an enhanced contribution by early progenitors to the maintenance of steady-state erythropoiesis (McDonagh 1992).

In in vitro cultures, effects of HU are dose and time; it appears to affect hemoglobin phenotype by direct interaction with relatively late erythroid precursors that are already engaged in hemoglobin production (Fibach 1993).
There is some evidence that HU may, in addition to inhibiting ribonucleoside diphosphate reductase, directly damage DNA and may inhibit the incorporation of thymidine into DNA (Dorr 1994, Young 1964), but this has not been proven.

1.2.4.5.2. In Vitro Studies

HU (25 – 400 μmol/L) was added to cultures derived from normal individuals and patients with Hb SS and β-thalassemia. In the cultures from normal donors, HU had an effect on erythroid cell numbers, MCH, mean cell volume (MCV), and Hb F, which was dependent on dose of HU and time of addition. Doses of less than 200 μmol/L had minimal effect on cell yield, no matter when it was added. On the other hand, 400 μmol/L of HU added at day four reduced cell yield by 90%. Increases in MCH and Hb F were observed with the addition of at least 100 μmol/L. Multiple administrations of HU led to a further increase in Hb F. In cells cultured at day 13, there was a 2.4-fold increase in Hb F in cultures receiving HU at day seven. The increase was 3.0-fold in cultures receiving HU at days seven and nine, and 3.4-fold in cultures receiving HU at days seven, nine, and eleven. In four cultures from patients with Hb SS, there was a mean increase in Hb F of 3.2-fold (2.2- to 5.1-fold). The increase in Hb F in four β-thalassemia patients' cultures was 3.5-fold (1.3- to 6.2-fold) (Fibach 1993). In all cultures, HU caused an increase in Hb F, MCV, and MCH, and an inhibition of cell proliferation.

1.2.4.5.3. Animal Studies

Treatment of an anemic baboon with 25 - 50 mg/kg/day HU did not increase Hb F synthesis or Hb F levels (DeSimone 1982).

Letvin examined the effect of HU on two monkeys who were rendered anemic through repeat phlebotomies. HU administration began at day 62 from beginning of phlebotomy, at which point total hemoglobin concentration had stabilized at 6.5 g/dL in both monkeys. F-cells and Hb F, which were not detectable before phlebotomy had stabilized at 13% and 3%, respectively in animal A and 20% and 5%, respectively in animal B. HU was given for 5 days at 50 mg/kg/day. There was no response in total hemoglobin or reticulocytes, though a transient and small increase was seen in F-cell and Hb F. Fourteen days after the first HU trial ended, HU was given again for five days, at double the initial dose. Responses in F-cells and Hb F were seen within five days. The F-cells doubled in number, and Hb F% tripled. Increase in Hb F was greater than the increase in F-cells, indicating that Hb F per F-cell increased (Letvin 1984).
1.2.4.5.4. Human Studies

Platt treated two patients with Hb SS with HU at 50 mg/kg/day for five days. These patients had an increase in F-retics within 24-72 hours after treatment start. There were no objective clinical improvements in either patient, although both Hb F and total hemoglobin concentration increased. In 1986, Dover reported on the use of HU in eight patients with Hb SS; treatment led to a 2-fold or greater increase in F-reticulocytes in four patients. A decrease in reticulocytes was observed in all eight patients (Dover 1986).

In 1990, McDonagh presented the first report of the effectiveness of HU in β-thalassemia. McDonagh treated seven patients (six transfusion-dependent; 1 transfusion-independent). There was no observed decrease in transfusion requirement in the transfusion-dependent patients, despite several months of therapy. However, the transfusion-independent patient, who was splenectomised, showed an increase in total hemoglobin concentration, from 4.6 g/dL at baseline to 6.6 g/dL after several weeks of treatment. Concurrent increases in MCV, MCH, and reticulocytes were observed (McDonagh 1990).

Hajjar (1994) reported the use of HU in three adults with β-thalassemia intermedia. One patient could not receive transfusions due to painful priapism episodes and the other two had developed severe alloantibodies. HU had a variable effect on total hemoglobin concentration and percent Hb F, but in all patients, a lag phase of two to three weeks was followed by an increase in total hemoglobin concentration of 1.1 - 3.3 g/dL. The hemoglobin concentration stabilized and then started to decrease; an additional increase in hemoglobin concentration was seen when HU dose was increased. Acute erythroid toxicity was seen in all patients, at doses that were generally tolerated in sickle cell patients (Hajjar 1994).

Arruda (1997) also reported a patient in whom transfusions were difficult due to the development of alloantibodies. The patient was treated with HU for 36 months, and received intermittent transfusions during the first 12 months. In the 24 months in which no transfusions were given, a hemoglobin concentration of 10.6 to 11.9 g/dL was maintained (Arruda 1997).

A patient with heterozygous Hb Lepore presented with severe anemia (Hb = 5.8 g/dL) and extramedullary hematopoietic masses in the spinal cord. Severe alloantibodies made transfusions impossible. Treatment with HU for six months resulted in an increase in total
hemoglobin concentration of 3.9 g/dL (to 9.7 g/dL) and an increase in Hb F from 86 to 94% (absolute value from 4.9 g/dL to 9.1 g/dL). There was a decrease in \( \alpha/\gamma \) globin chain synthesis ratio from 3.5 at baseline to 2.4 after treatment. The extramedullary masses decreased with three months of treatment (Rigano 1997). A second patient in whom HU was used to reduce extramedullary masses was reported by Saxon et al., who treated a patient for 10 months. Total hemoglobin concentration increased from 7.0 - 7.5 g/dL to 9.0 - 10.0 g/dL, while absolute Hb F increased from 5.0 g/dL to 7.6 g/dL. As in the patient reported by Rigano, there was a regression of the paraspinal extramedullary hematopoiesis, as evaluated by magnetic resonance imaging (Saxon 1998).

The earlier studies were short-term. In 1995, Voskaridou reported 14 adults with Hb S/\( \beta \)-thalassemia who had received over 100 weeks of treatment. An initial induction phase of 15 mg/kg/day for 4 days/week, escalated every four weeks to maximum tolerated dose or 2.5 g/day lasted for 24 - 35 weeks. A significant increase in Hb F was seen in the induction period. Increases in MCV and MCHC (mean cellular hemoglobin concentration) were also observed. These changes were positively correlated to observed increases in percent Hb F. The correlation between increases in MCV and percent Hb F was 0.698 (P=0.054). A maintenance dose of 1 g/day for 4 days/week was then given for 12 weeks. During the maintenance period, percent Hb F returned to baseline levels (Voskaridou 1995).

Thalassemia intermedia patients treated with HU showed a quick increase in Hb F, which increased two- to four-times baseline within three months of treatment. Further increases in HU did not lead to a greater Hb F response. Although patients reported a subjective clinical improvement, there was no increase in total hemoglobin concentration (Loukopoulos 1998). Styles et al. treated four transfusion-independent patients (1 \( \beta \)-thalassemia intermedia, 3 Hb E/\( \beta \)-thalassemia) with low doses of HU (10 - 15 mg/kg/day). Three patients responded with increases in total hemoglobin concentration of 1 - 2 g/dL. Modest increases in Hb F were reported for two of the responders (Styles 1998).

Significant variations in both the timing and magnitude of Hb F stimulation have been observed between each study, and within each study (Stamatoyannopoulos 1992). Studies appear to indicate that the use of HU as a single agent in patients with \( \beta \)-thalassemia will likely not prove effective (Dover 1998, McDonagh 1990, Olivieri 1996, Stamatoyannopoulos 1992).
1.2.4.6. Combination Therapy

Studies in baboons showed that administration of HU and EPO together elevated F-reticulocytes to a greater extent than either drug alone (Al-Khatti 1988, DeSimone 1982). The same is true for the co-administration of HU and SPB (McDonagh 1992).

Although studies in patients with sickle cell anemia reported an additive effect with the combination treatment of HU and EPO (Goldberg 1990), the results in β-thalassemia have not shown this effect in most patients. Of ten thalassemia intermedia patients treated with a combination of HU and EPO, eight showed an increase in total hemoglobin concentration of 1.7 ± 1.4 g/dL. The responders were then randomly assigned to one of four arms: high dose EPO with or without HU or low dose EPO with or without HU. Only those who received high dose EPO continued to show a response. The presence or absence of HU did not change this response (Loukopoulos 1998). All patients reported a clinical improvement.

In a second study with β-thalassemia intermedia patients, all patients had an increase in total hemoglobin concentration with each drug individually and this increase was only improved by the combined administration in two of seven patients. No increase in Hb F was seen with EPO alone, while HU gave an increase in Hb F and an improvement in red cell indices with or without the administration of EPO (Aker 1994).

Recently, Hoppe treated two Hb E/β-thalassemia patients with HU and SPB. Treatment with HU alone increased baseline hemoglobin levels of 8.1 g/dL and 7.1 g/dL to 11.5 g/dL and 7.7 g/dL, respectively. There were concurrent increases in Hb F from 32% to 58% in patient 1 and from 33% to 39% in patient 2. The addition of SPB led to further increases in Hb F to 62% and 53%, respectively, but there were no increases in total hemoglobin concentration in either patient (Hoppe 1999).

In the chicken, the ρ-globin gene acts in a similar manner to the γ-globin gene in humans; both are the predominant non-α globin in the fetal stage, but are essentially turned off in the adult stage. Sites in the 5' upstream putative control regions of the ρ-globin in the chicken are highly methylated in adult chicken reticulocytes, but completely unmethylated in 5-day embryonic red cells. Treatment with 5-azaC results in demethylation around the ρ-globin gene. RNA isolated from reticulocytes of treated chickens showed small amounts of ρ-globin RNA, while RNA
isolated from controls showed no β-globin RNA. When SPB was given following treatment with 5-azaC, there was an increase in the amount β-globin RNA seen. SPB, given without pre-treatment by 5-azaC, did not appear to induce β-globin RNA (Ginder 1984).

1.2.5. Splenectomy

Due to the incidence of hypersplenism and red cell sequestration seen in patients with β-thalassemia, splenectomy has been used to increase baseline hemoglobin concentration and avoid the need for regular transfusions in patients who have β-thalassemia intermedia, but are exhibiting a decrease in hemoglobin concentration.

1.2.5.1. Proposed Hematological Benefits of Splenectomy

Hematological benefit post of splenectomy is generally attributed to two factors: reduction in plasma volume, and removal of a site of red cell destruction (Nightingale 1972).

Blendis reported seven patients who were splenectomised; of the four patients who were transfusion-dependent prior to splenectomy, two became transfusion-independent and one patient had a reduction in transfusion requirement. A moderate rise in hemoglobin of 2.7 (1.7 – 5.6) g/dL was reported for four of the patients, all of whom had exhibited significant pooling of red cells in the spleen. In three of the five patients studied, a significant reduction in the degree of ineffective erythropoiesis was reported. Clinical improvement was reported in all patients; they reported improved appetite and ‘well being’. In addition, three patients reported growth spurts following splenectomy (Blendis 1974). Contrarily, in an analysis of growth in patients with β-thalassemia, Logothetis reported that patients who were splenectomised (n=56) were shorter than the non-splenectomised patients (n=82). The splenectomised patients were -1.35 and the non-splenectomised patients were -0.79 standard deviations below normal for age (P=0.01) (Logothetis 1972). However, the splenectomised patients likely represented those with a more severe illness and their short stature may not be a direct result of the splenectomy.

In a report of 18 patients with β-thalassemia intermedia, Fiorelli et al. reported eight who were splenectomised for ‘increasing splenomegaly leading to hypersplenism’. Of these eight patients, splenectomy performed at a mean age of 12 (4 – 26) years.
for 'worsening anemia'. Of these, eight avoided the initiation of regular red cell transfusions (Pippard 1982). In the same study, all transfusion-dependent patients over the age of seven had been splenectomised, at a mean age of 6 (2 – 10) years due to increasing blood transfusion requirements and showed a varying decrease in transfusion needs following splenectomy.

Engelhard conducted a retrospective review of 30 patients who had undergone splenectomy. These patients were divided into three groups according to the severity of disease. The 16 patients who had required regular transfusions by the age of two showed only a temporary hematological improvement of one to two years. The eight patients who required regular transfusions only after several years showed a response that lasted for at least three years. In six patients with β-thalassemia intermedia, who were mainly splenectomised due to mechanical pressure from splenomegaly, continued improvement in hemoglobin concentration was observed (Engelhard 1975). He reported no increase in the incidence or severity of infection after splenectomy in any the entire population. Five patients had infections post-splenectomy while two had had infections while the spleen was intact (P=0.42). All patients, regardless of hematological response, showed a clear spurt in growth and development following surgery.

1.2.5.2. Possible Risks of Splenectomy

The removal of the spleen removes a blood filter, although the liver is able to perform this function to some extent (Crosby 1963, Pearson 1995). Without splenic culling and pitting, the number of nRBC, and cells with inclusion bodies increases significantly (Fessas 1963, Pearson 1995). People who have had their spleens removed, for whatever reason, may be more prone to infections and thromboembolic complications.

The removal of the spleen allows very deformed cells to remain in circulation. Dondorp compared the elongation index, which is a unit of deformability, in splenic and asplenic β-thalassemia patients. He found that, at a shear stress equivalent to that seen in the spleen (30 Pa), the red cell deformability of the asplenic patients was significantly lower than those of the non-splenectomised patients; all asplenic patients exhibited red cell deformabilities below 0.45, while all non-splenectomised patients were above 0.45 (Dondorp 1999). This indicates that the removal of the spleen allows the continued circulation of very abnormal red cells.
1.2.5.2.1. Post-Splenectomy Sepsis

The incidence of mortality due to sepsis in the general population is 0.01% (Singer 1973). Thalassemic children are particularly susceptible to fatal gram-positive septicemia post-splenectomy (Bunn 1986). In 1973, Singer conducted meta-analysis of 24 series of patients from literature, which included 2,795 patients who were splenectomised for various reasons. He divided the reasons for splenectomy into one of nine categories, including thalassemia, trauma, and congenital spherocytosis; 109 patients had β-thalassemia. Of the 2,795 patients, 119 (4.25%) developed post-splenectomy sepsis, and 71 (2.52%) died as a result of their infection. Of the 109 patients with thalassemia in this study, 27 (24.8%) developed post-splenectomy sepsis, and 12 (11.0%) consequently died. Of the nine disease categories studied, thalassemia patients had a significantly higher rate of morbidity and mortality due to sepsis than did the other; when compared to the general population, asplenic thalassemic patients were 1,100 times more likely to develop sepsis (Singer 1973). Singer repeated this analysis in 2001 with a total of 7872 patients. In this recent analysis, the overall rate of sepsis was 3.5%, a decrease of 18% from the 1973 study; while the rate of fatal sepsis was 2.1%, a decrease of 16% from the 1973 study. Again, patients with β-thalassemia (265 patients in this analysis) had the highest rates of both sepsis and fatal sepsis; 13.2% and 5.6%, respectively (Hansen 2001).

Smith et al. reported 55 patients followed at the New York Hospital between 1944 and 1961, 33 of who had been splenectomised. The splenectomised cohort had seven severe infections that were fatal in five cases. The non-splenectomised cohort (which included the splenectomised patients before their splenectomy) saw three severe infections, none of which were fatal (Smith 1962). Analysis of these two groups indicates a significant increase in severe infections in the splenectomised group (P=0.036), and a significant increase in death from infections (P=0.005).

A review of published reports described 460 children splenectomised for various causes, 14 of whom had β-thalassemia (Broberger 1960). It indicated that the risk of severe infections was higher in those patients splenectomised at less than one year of age. In these younger patients, 13 of 58 (22.4%) sustained severe infections, while the rate was 10.6% in the other patients (Broberger 1960). Singer reported a similar correlation between a young age at splenectomy and the occurrence of post-splenectomy sepsis; he reported that 75% of the severe infections in recorded cases occur within the first two years post-splenectomy and almost 50% occur within the first 12 months (Singer 1973). The greatest risk of infection occurs in infants and young children, and is generally due to encapsulated organisms (Hansen 2001).
Antibody response to some antigens in asplenic individuals is about one-tenth of the response seen in people with spleens (Hansen 2001). Infection in splenectomised patients can be reduced through adequate prophylactic therapy including vaccination against Pneumococcus pneumoniae, which is the most frequent sepsis in these patients (Piomelli 1995). Jugenburg et al. compared the rate of post-splenectomy infection in patients splenectomised between 1958 and 1970, who received no prophylactic therapy; with those splenectomised between 1971 and 1975, who all received prophylactic antibiotics and two-thirds of whom received polyvalent pneumococcal vaccines. In the later cohort, there were 47% fewer post-splenectomy infections, and an 88% decrease in mortality from overwhelming sepsis (Jugenburg 1999). Although the incidence of post-splenectomy infections has decreased, one of seven patients with β-thalassemia will develop post-splenectomy sepsis. This is the highest rate for post-splenectomy sepsis in any hematological disease (Hansen 2001).

1.2.5.2.2. Pulmonary Changes Following Splenectomy
The normal pulmonary blood flow is 5 liters per minute at rest. Obstructing thrombotic emboli decrease the functional cross-sectional area of the vascular bed, increasing pulmonary resistance. The pulmonary arterial pressure and right arterial pressure increase in an effort to increase the amount of blood flowing through the functional parts of the vascular bed.

In 1966, Hirsh and Dalcie reported post-splenectomy thrombotic complications in some patients who had undergone the surgery (Hirsh 1966). They conducted a retrospective analysis of 80 patients who had been splenectomised for various reasons, including hereditary spherocytosis, Heinz body anemia, elliptocytosis, and thalassemia. Post-splenectomy platelet counts were elevated in all patients with persistent anemia (hereditary non-spherocytic anemia, sideroblastic anemia and thalassemia), while those patients who were hematologically normal had normal platelet counts and those with hereditary spherocytosis or elliptocytosis had normal or minimally raised platelet counts. Thus, platelet count was negatively correlated to hemoglobin concentration (P<0.001; r² not given). This correlation was not seen in non-splenectomised patients with the same disorders (P>0.1; r² not given). Similarly, a correlation was seen between thrombocytosis and reticulocytosis in splenectomised (P<0.001; r² not given) but not non-splenectomised (P>0.1; r² not given). In an example given of one β-thalassemia major patient, there was no correlation between platelets and hemoglobin pre-splenectomy, but post splenectomy any changes in hemoglobin concentration caused a reciprocal change in platelet
count. This correlation was not a result of thalassemia, but rather of the anemia. A red-cell aplasia patient, with a normal or slightly elevated platelet count pre-splenectomy had a sharp rise in platelet count post-splenectomy. The thrombocytosis persisted for nine years, until the anemia corrected, at which point the platelet count returned to normal (Hirsh 1966).

Hoeper conducted a retrospective review of all patients treated at the Hannover Medical School in Hannover, Germany for idiopathic pulmonary hypertension between 1993 and 1999. Of the 61 patients seen, seven had had splenectomies. None of these patients had thalassemia; the splenectomies were done for spherocytosis (n=3), ITP (n=1), and trauma (n=3). Pulmonary hypertension developed 4 to 34 years after splenectomy. Three of these patients underwent lung transplantation, allowing examination of the explanted lungs. All showed evidence of lung disease including medial hypertrophy, marked medial thickening, plexiform lesions, and multiple thrombotic lesions. These thrombotic lesions are not usually seen in pulmonary hypertension indicating that the splenectomy-induced idiopathic hypertension may have an unique etiology. One of these three patients underwent a second lung transplant, 5 years after the first, due to a recurrence of pulmonary hypertension; recurrence of pulmonary hypertension following transplant had not previously been reported. Examination of the explanted graft showed intimal fibrosis, disseminated occlusion of small pulmonary arteries, and extensive thrombotic lesions. Hoeper compared the incidence of splenectomy in those patients presenting with idiopathic pulmonary hypertension and those presenting with other lung diseases and found a significant difference (P<0.0001) between the two groups (cystic fibrosis, emphysema, pulmonary fibrosis, and Eisenmenger syndrome). Seven of the 61 patients with idiopathic pulmonary hypertension (11.5%; 95% CI: 4.7 – 22.2%) and none of the 151 other patients (0%; 95% CI: 0.0 – 3.2%) had had splenectomies. Hoeper estimated that 15,000 splenectomies, 0.02% of the population, were performed in Germany each year. Thus, in a mixed population with a mean age of 50 years, one would expect to see a splenectomy rate of about 1.0%, instead of the 11.5% that was observed in the patients treated for idiopathic pulmonary hypertension (Hoeper 1999).

1.3. Statistical Analysis
Comparison of baseline parameters to peak or post treatment parameters was done with a paired student’s T-test, which compares the difference in measurements within each pair (Pagano 2000).
In comparing the responders to the others, a two-sample t-test was utilized for continuous variables. This test compares the mean value of one population to the mean value in a second population (Pagano 2000).

For parameters reported as proportions (e.g., transfusion-dependence) Fisher's exact analysis was used (Pagano 2000).
Chapter 2

Splenectomy in Thalassemia Intermedia

2.1. Background

Removal of a large, hypersplenic spleen leads to a reduction in plasma volume and the removal of the main site of red cell destruction (Nightingale 1972). Therefore, in transfusion-independent β-thalassemia patients, splenectomy will, theoretically, provide an increase in hemoglobin concentration. The effectiveness of splenectomy as a non-transfusion approach β-thalassemia has not been adequately analyzed, although anecdotal evidence suggests that response may be variable (Engelhard 1975, Fiorelli 1987, Pippard 1982).

The adverse effects reported from splenectomy include increased infections and pulmonary hypertension, the former due to the removal of the immunological functions of the spleen and the latter due to thrombic emboli from persistent thrombocytopenia. The intent of this thesis was to retrospectively determine if splenectomy has an effect on hemoglobin concentration or clinical parameters. Since there has been significant variability reported in response to splenectomy, this study also sought to determine what baseline parameters, if any, could be used to predict response. Furthermore, the incidence of adverse effects attributable to splenectomy, including infections and pulmonary hypertension were also examined.

2.2. Analysis Pre- and Post-Splenectomy

2.2.1. Purpose of Study

A retrospective study on the effects of splenectomy in patients with β-thalassemia intermedia was conducted by comparing patients pre- and post-splenectomy. The purposes of this study were: (1) to evaluate the impact of splenectomy on hemoglobin concentration; (2) to evaluate the impact of splenectomy on height velocity in patients splenectomised as children; and (3) to determine which baseline parameters, if any, can be used to predict response.

We hypothesized that splenectomy would improve hemoglobin concentration in all patients and height velocity in children. We further hypothesized that baseline parameters, including spleen size, age, and baseline hemoglobin concentration could help to predict response.
2.2.2. Experimental Design

The charts of all patients followed at the Toronto Hospital for Sick Children or the Toronto General Hospital, who are or were transfusion-independent, were reviewed to locate patients who received a splenectomy while transfusion-independent. To be considered evaluable, pre-splenectomy hematological values were needed, as well as post-splenectomy values for at least 12 months following surgery. Baseline hematological parameters were taken as the mean of all visits in the 12 months prior to splenectomy. One patient was not regularly seen in clinic, and the three visits immediately prior to splenectomy were over 29.2 months. One patient was followed for only 8.5 months pre-splenectomy; the 13 visits recorded over this period were taken as baseline. Two patients had only two visits prior to splenectomy, and therefore only two visits were meaned for these patients. Spleen size was taken as the measurement closest to splenectomy. The number of transfusions pre-splenectomy was determined through chart analysis. Transfusions in outside centers were included for two patients and were determined through patient histories and consult notes. Post-splenectomy hematological parameters were a mean of the twelve months immediately following splenectomy. For those patients who received a transfusion at splenectomy (n=8), the twelve months of follow-up started three months after the date of transfusion. In all cases (pre- and post-splenectomy), no hematological parameters were included for three months post-transfusion. Long-term follow-up was defined as the most recent visit in those patients that remained transfusion-independent; for those patients who began regular transfusions (n=2) or underwent BMT (n=1), the visit before intervention was defined as the date of long-term follow-up. Height velocity pre-splenectomy was determined in the year immediately prior to splenectomy; height velocity post-splenectomy was determined in the year immediately post-splenectomy. Response was defined as an increase in total hemoglobin concentration greater than or equal to 1.5 g/dL over those obtained at baseline. Comparison of baseline parameters in the responder vs. others was done through unpaired Student's T-test.
2.2.2.1. Patient Population

Figure 2.1 Eligibility of Thalassemia Intermedia patients for pre- and post-splenectomy comparison analysis

Chart review uncovered 44 patients diagnosed with β-thalassemia intermedia of whom 21 received splenectomies while transfusion-independent; ten of these patients were evaluable in this study. Of the remaining eleven patients, nine were splenectomised in other centers before being followed in Toronto, one began regular transfusions less than twelve months post-splenectomy, and one had been splenectomised less than twelve months before this study. This is displayed graphically as figure 2.1.

The ten eligible patients included eight females and two males. Clinical characteristics are shown in table 2.1. The mean age at splenectomy was 9.6 ± 2.5 (4.2 - 28.2) years, with a mean post-splenectomy follow-up of 10.3 ± 3.2 (1.0 - 33.8) years. Current age is 22.0 ± 3.8 (6.2 - 40.8) years.

Eight of the patients received occasional transfusions prior to splenectomy; 3.4 ± 0.8 (1 - 8) transfusions in these eight patients. The mean spleen size at splenectomy was 9.7 ± 1.5 (3 - 19) cm. The hematological parameters at baseline are shown in table 2.2. Mean hemoglobin
concentration was $6.4 \pm 0.3 \ (4.8 - 7.5) \ g/dL$, platelet concentration was $252.3 \pm 32.3 \ (101.5 - 372.3) \times 10^9/L$, WBC was $8.2 \pm 1.1\ (5.1 - 14.9) \times 10^9/L$, and nRBC were $17.6 \pm 5.2 \ (2 - 52)/100 \ WBC$.

**Table 2.1** Clinical characteristics of $\beta$-thalassemia intermedia patients analyzed pre- and post-splenectomy (n=10)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at Splenectomy (yrs)</th>
<th>Previous Transfusions</th>
<th>Spleen size at Splenectomy (cm)</th>
<th>Current Age (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RU</td>
<td>F</td>
<td>4.2</td>
<td>4</td>
<td>3.0</td>
<td>10.1</td>
</tr>
<tr>
<td>IC</td>
<td>F</td>
<td>4.3</td>
<td>4</td>
<td>13.0</td>
<td>12.5</td>
</tr>
<tr>
<td>SO</td>
<td>F</td>
<td>4.5</td>
<td>0</td>
<td>9.0</td>
<td>13.3</td>
</tr>
<tr>
<td>NK</td>
<td>F</td>
<td>5.2</td>
<td>0</td>
<td>19.0</td>
<td>6.2</td>
</tr>
<tr>
<td>WT</td>
<td>F</td>
<td>5.7</td>
<td>5</td>
<td>12.0</td>
<td>17.5</td>
</tr>
<tr>
<td>JR</td>
<td>M</td>
<td>5.7</td>
<td>1</td>
<td>7.0</td>
<td>40.8</td>
</tr>
<tr>
<td>JN</td>
<td>F</td>
<td>7.2</td>
<td>2</td>
<td>11.0</td>
<td>24.4</td>
</tr>
<tr>
<td>FL</td>
<td>F</td>
<td>12.3</td>
<td>1</td>
<td>8.0</td>
<td>23.9</td>
</tr>
<tr>
<td>MM</td>
<td>M</td>
<td>18.4</td>
<td>8</td>
<td>10.0</td>
<td>34.2</td>
</tr>
<tr>
<td>CD</td>
<td>F</td>
<td>28.2</td>
<td>2</td>
<td>3.5</td>
<td>37.5</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>9.6</td>
<td>2.7</td>
<td>9.7</td>
<td>22.0</td>
</tr>
</tbody>
</table>

**Table 2.2** Baseline hematological characteristics of $\beta$-thalassemia intermedia patients analyzed pre- and post-splenectomy (n=10)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline Hb (g/dL)</th>
<th>Baseline WBC ($\times 10^9$/L)</th>
<th>Baseline Platelets ($\times 10^9$/L)</th>
<th>Baseline nRBC/100 WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>4.8</td>
<td>9.7</td>
<td>156.1</td>
<td>36.5</td>
</tr>
<tr>
<td>WT</td>
<td>5.3</td>
<td>5.1</td>
<td>341.5</td>
<td>3.0</td>
</tr>
<tr>
<td>FL</td>
<td>5.5</td>
<td>6.6</td>
<td>357.0</td>
<td>11.0</td>
</tr>
<tr>
<td>IC</td>
<td>6.5</td>
<td>10.3</td>
<td>236.2</td>
<td>24.6</td>
</tr>
<tr>
<td>MM</td>
<td>6.6</td>
<td>6.2</td>
<td>365.0</td>
<td>2.0</td>
</tr>
<tr>
<td>RU</td>
<td>6.7</td>
<td>14.9</td>
<td>372.3</td>
<td>52.3</td>
</tr>
<tr>
<td>JR</td>
<td>7.1</td>
<td>5.3</td>
<td>101.5</td>
<td>2.0</td>
</tr>
<tr>
<td>CD</td>
<td>7.2</td>
<td>7.1</td>
<td>132.3</td>
<td>17.5</td>
</tr>
<tr>
<td>JN</td>
<td>7.3</td>
<td>5.2</td>
<td>221.5</td>
<td>7.0</td>
</tr>
<tr>
<td>SO</td>
<td>7.5</td>
<td>11.2</td>
<td>239.2</td>
<td>19.8</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.4</td>
<td>8.2</td>
<td>252.3</td>
<td>17.6</td>
</tr>
</tbody>
</table>

2.2.3. Hematological Changes Following Splenectomy

Five of the ten patients exhibited a response, defined as an increase in hemoglobin concentration equal to or greater than 1.5 g/dL; while, the remaining five patients exhibited no or a non-significant response.

The five responders had a mean increase in hemoglobin of $2.7 \pm 0.4 \ (2.0 - 4.1) \ g/dL$; from $6.7 \pm 0.3 \ (5.5 - 7.5) \ g/dL$ at baseline to $8.9 \pm 0.7 \ (6.8 - 10.7) \ g/dL$ during the first twelve months post-
splenectomy. All five of the responders remained transfusion-independent, 15.7 ± 5.3 (1.0 – 33.8) years after splenectomy. At their most recent visit, mean hemoglobin concentrations in these five patients was 8.3 ± 0.7 (6.9 – 10.6) g/dL which is 2.1 ± 0.6 (0.3 – 4.0) g/dL over hemoglobin concentrations determined pre-splenectomy.

The other five patients changed from a hemoglobin concentration of 6.2 ± 0.5 (4.8 – 7.3) g/dL pre-splenectomy to 6.7 ± 0.5 (4.9 – 7.9) g/dL post-splenectomy, for a mean change of 0.5 ± 0.4 (-1.6 – 1.1) g/dL in the first 12 months. Two patients began regular red cell transfusions, 1.8 and 2.1 years after splenectomy; one underwent a BMT 2.8 years after splenectomy; and two remain transfusion-independent, 7.1 and 11.1 years post-splenectomy. Mean hemoglobin concentration at the most recent evaluable visit (or prior to initiation of therapy) was 7.0 ± 0.6 (5.2 – 8.8) g/dL; an increase of 0.4 ± 0.8 (-1.3 – 3.3) g/dL over that determined pre-splenectomy.

The hematological changes observed in the five responders are shown in table 2.3, while those of the five others are in table 2.4. Figure 2.2 depicts the change in hemoglobin concentration over the first twelve months post-splenectomy in all ten patients with the responders in blue and the non-responders in red. The yellow line at 1.5 g/dL of hemoglobin represents the threshold of response.

Table 2.3  Hemoglobin concentrations at baseline, 12 months post-splenectomy, and at follow up for β-thalassemia intermedia patients with an increase in Hb ≥ 1.5 g/dL following splenectomy (n=5)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline Hb (g/dL)</th>
<th>Hb in 1st 12 months (g/dL)</th>
<th>Change in Hb 1st 12 months (g/dL)</th>
<th>Recent Hb (g/dL)</th>
<th>Change from baseline to most recent Hb (g/dL)</th>
<th>Follow up (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>4.8</td>
<td>6.8</td>
<td>2.0</td>
<td>6.9</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>WT</td>
<td>5.3</td>
<td>7.5</td>
<td>2.3</td>
<td>7.2</td>
<td>1.9</td>
<td>11.6</td>
</tr>
<tr>
<td>MM</td>
<td>6.6</td>
<td>10.7</td>
<td>4.1</td>
<td>10.6</td>
<td>4.0</td>
<td>15.5</td>
</tr>
<tr>
<td>JR</td>
<td>7.2</td>
<td>10.1</td>
<td>2.9</td>
<td>9.1</td>
<td>1.9</td>
<td>33.8</td>
</tr>
<tr>
<td>JN</td>
<td>7.3</td>
<td>9.5</td>
<td>2.2</td>
<td>7.6</td>
<td>0.3</td>
<td>16.5</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.2</td>
<td>8.9</td>
<td>2.7</td>
<td>8.3</td>
<td>2.1</td>
<td>15.7</td>
</tr>
</tbody>
</table>
As shown in Table 2.5, there were no significant differences at baseline between those who showed a response and those who did not show a response. Both groups had similar transfusion histories, $3.2 \pm 1.5$ (0 – 8) transfusions in the responders vs. $2.2 \pm 0.8$ (0 – 4) transfusions in the others, $P=0.57$; spleen sizes, $12.0 \pm 1.9$ (8 – 19) cm in the responders vs. $7.3 \pm 1.9$ (3 – 13) cm in the others, $P=0.11$; and age at splenectomy, $8.4 \pm 2.5$ (5.2 – 18.4) years in the responders vs. $10.8 \pm 4.7$ (4.2 – 28.2) years in the others, $P=0.67$.

Similarly, there were no significant differences in baseline hematological parameters between the responders and the others. Baseline hemoglobin concentration was $6.2 \pm 0.5$ (4.8 – 7.3) g/dL in those who showed a response and $6.2 \pm 0.5$ (4.8 – 7.3) g/dL in those who did not show a response. Both groups had similar transfusion histories, $3.2 \pm 1.5$ (0 – 8) transfusions in the responders vs. $2.2 \pm 0.8$ (0 – 4) transfusions in the others, $P=0.57$; spleen sizes, $12.0 \pm 1.9$ (8 – 19) cm in the responders vs. $7.3 \pm 1.9$ (3 – 13) cm in the others, $P=0.11$; and age at splenectomy, $8.4 \pm 2.5$ (5.2 – 18.4) years in the responders vs. $10.8 \pm 4.7$ (4.2 – 28.2) years in the others, $P=0.67$.

Table 2.4  Hemoglobin concentration at baseline, 12 months post-splenectomy, and at follow up for β-thalassemia intermedia patients with an increase in Hb < 1.5 g/dL following splenectomy (n=5)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline Hb (g/dL)</th>
<th>Hb in 1st 12 months (g/dL)</th>
<th>Change in Hb 1st 12 months (g/dL)</th>
<th>Recent Hb (g/dL)</th>
<th>Change from baseline to most recent Hb (g/dL)</th>
<th>Follow-up (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>6.5</td>
<td>4.9</td>
<td>-1.6</td>
<td>5.2</td>
<td>-1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>FL</td>
<td>5.5</td>
<td>6.6</td>
<td>1.1</td>
<td>8.8</td>
<td>3.3</td>
<td>11.1</td>
</tr>
<tr>
<td>RU</td>
<td>6.7</td>
<td>7.1</td>
<td>0.4</td>
<td>6.6</td>
<td>-0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>CD</td>
<td>7.2</td>
<td>7.1</td>
<td>-0.3</td>
<td>7.7</td>
<td>0.5</td>
<td>7.1</td>
</tr>
<tr>
<td>SO</td>
<td>7.5</td>
<td>7.9</td>
<td>0.4</td>
<td>6.9</td>
<td>-0.6</td>
<td>2.8</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.7</td>
<td>6.7</td>
<td>0.1</td>
<td>7.0</td>
<td>0.4</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Figure 2.2  Change in Hb (g/dL) from baseline to 12 months post-splenectomy in β-thalassemia intermedia patients analyzed pre- and post-splenectomy (n=10)
g/dL in the responders and 6.7 ± 0.3 (5.5 – 7.5) g/dL in the others; \( P=0.45 \). Baseline WBC was 6.3 ± 0.9 (5.1 – 9.8) \( \times 10^9/L \) in the responders and 10.1 ± 1.5 (6.6 – 14.9) \( \times 10^9/L \) in the others, \( P=0.061 \). Baseline platelet concentrations were 237.1 ± 51.2 (101.5 – 365.0) \( \times 10^9/L \) and 267.4 ± 44.2 (132.3 – 372.3) \( \times 10^9/L \) respectively, \( P=0.67 \). The responders had 10.1 ± 6.7 (2 – 37) nRBC/100 WBC vs. 25.1 ± 7.2 (11 – 52) nRBC/100 WBC in the others, \( P=0.16 \).

Table 2.5 Comparison of baseline parameters in \( \beta \)-thalassemia intermedia patients who responded to splenectomy (n=5) and those who did not (n=5)

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Others</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Age at splenectomy (yrs)</td>
<td>8.4 ± 2.5</td>
<td>10.8 ± 4.7</td>
<td>0.67</td>
</tr>
<tr>
<td>Number of transfusions prior to splenectomy</td>
<td>3.2 ± 1.5</td>
<td>2.2 ± 0.8</td>
<td>0.57</td>
</tr>
<tr>
<td>Spleen size (cm)</td>
<td>12.0 ± 1.9</td>
<td>7.3 ± 1.9</td>
<td>0.11</td>
</tr>
<tr>
<td>Baseline Hb (g/dL)</td>
<td>6.2 ± 0.5</td>
<td>6.7 ± 0.3</td>
<td>0.45</td>
</tr>
<tr>
<td>Baseline WBC ( \times 10^9/L )</td>
<td>6.3 ± 0.9</td>
<td>10.1 ± 1.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Baseline platelets ( \times 10^9/L )</td>
<td>237.1 ± 51.2</td>
<td>267.4 ± 44.2</td>
<td>0.67</td>
</tr>
<tr>
<td>Baseline nRBC/100 WBC</td>
<td>10.1 ± 6.7</td>
<td>25.1 ± 7.2</td>
<td>0.16</td>
</tr>
</tbody>
</table>

2.2.4. Changes in Linear Growth Following Splenectomy

Six patients were splenectomised as children, and therefore a comparison in height velocity pre- and post-splenectomy could be made.

As is shown in table 2.6, with the exception of one outlier (IC), those who had a better hematological response also showed a greater growth spurt following splenectomy.

Table 2.6 Change in height velocity (%ile) post-splenectomy in \( \beta \)-thalassemia intermedia patients splenectomised as children (n=6)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Height Velocity (%ile)</th>
<th>Change in Hb (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>17.5</td>
<td>75</td>
</tr>
<tr>
<td>SO</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>RU</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>NK</td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>JN</td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td>WT</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>MEAN</td>
<td>16.4</td>
<td>61.5</td>
</tr>
</tbody>
</table>

As demonstrated in figure 2.3, due to the small number of patients and the fact that IC is a significant outlier, no real correlation was observed between change in total hemoglobin concentration and change in height velocity \( (r^2=0.48, P=0.33) \). If IC is removed from the
analysis, a significant correlation of $r^2=0.97$ with $P=0.002$ is found. A careful review of the hospital chart yielded no reasons for IC's significant growth spurt.

Figure 2.3  Change in Hb (g/dL) vs. change in height velocity (%ile) post-splenectomy in β-thalassemia intermedia patients splenectomised as children

2.2.5. Comments on analysis pre- and post-splenectomy in patients with β-thalassemia intermedia

Based on the data collected, the efficacy of splenectomy in β-thalassemia intermedia remains unclear. Five of the ten patients showed an increase in total hemoglobin concentration of at least 1.5 g/dL over the first twelve months post-splenectomy; an equal number of patients showed either no, or a non-significant, response. Other studies have reported a similar variation in response to splenectomy (Fiorelli 1987, Modell 1984, Pippard 1982), although a report by Engelhard included five β-thalassemia intermedia patients, all of who showed a sustained increase in hemoglobin concentration (Engelhard 1975).

Due to the variation seen in response, it would be helpful if there were baseline parameters that could be used to predict response. However, comparison of baseline parameters did not elucidate any significant differences between the responders and the others. Since splenectomy is proposed to be beneficial by reducing plasma volume and removing a site of red cell destruction (Nightingale 1972), the extent of hypersplenism may be correlated to response. However, as noted by Piomelli (1995), spleen size does not indicate the severity of hypersplenism; and spleen size was not correlated to response in our patients. Similarly, no correlation to response was seen in baseline hemoglobin, age at splenectomy, or number of transfusions pre-splenectomy.
Of the ten patients analyzed, seven remain transfusion-independent. Of the other three, all of whom were non-responders, two began regular transfusions 1.8 and 2.1 years post-splenectomy and one patient underwent BMT after 2.8 years. The proportion of patients who have avoided the initiation of regular therapy (70%) is slightly higher than that of other reports, which range from 25% to 50% (Fiorelli 1987, Pippard 1982). However, it may be that our center has more stringent requirements for the initiation of regular red cell transfusions. Secondly, the patients reported by others may have been more severely affected by disease, since Engelhard reported that splenectomy appeared to be more effective in patients with milder clinical courses (Engelhard 1975).

As reported by others (Blendis 1974, Engelhard 1975), a growth spurt was observed in most patients splenectomised as children. However, unlike the report by Engelhard, with one exception, increases in growth velocity appeared to be correlated to increases in hemoglobin concentration. The outlier is a patient who did not exhibit a hematological response (change in hemoglobin concentration was $-1.6 \text{ g/dL}$), but had an increase in height velocity from the 17.5 percentile pre-splenectomy to the 75 percentile post-splenectomy. A careful review of this patient’s hospital chart did not suggest any reasons for this growth spurt.

Hirsh and Dalcie reported that the post-splenectomy rise in platelets was inversely proportional to the level of anemia post-splenectomy (Hirsh 1966). However, although all patients exhibited an increase in platelet count following splenectomy, this was not correlated to hemoglobin concentration in our patients.

Only one patient reported an adverse effect due to splenectomy; this patient (NK), a responder, developed thrombotic complications.

In our center, splenectomy is now rarely performed in $\beta$-thalassemia intermedia patients, as there is no conclusive evidence that this invasive intervention provides a significant clinical benefit. Unfortunately, this analysis provides no concrete conclusions to aid in the decision to splenectomise a $\beta$-thalassemia intermedia patient.
2.3. Comparison of Splenectomised and Non-Splenectomised β-Thalassemia Intermedia Patients

2.3.1. Purpose of Study
The effect of splenectomy on patients with β-thalassemia intermedia was also studied by comparing the splenectomised patients to the non-splenectomised patients. The purpose of this study was to compare a cohort of splenectomised β-thalassemia intermedia patients with a cohort of non-splenectomised β-thalassemia intermedia patients to determine the effects of splenectomy on: (1) hematological parameters; (2) clinical characteristics; and (3) growth.

2.3.2. Experimental Design
The charts of all patients followed at the Toronto Hospital for Sick Children or the Toronto General Hospital, who are or were transfusion-independent, were reviewed. Only patients who displayed true thalassemia intermedia phenotypes (at least four years of transfusion-independence) were included in the analysis. Hematological parameters were taken from three visits divided over the most recent twelve months. For patients that did not have three visits within twelve months (n=10), older records were reviewed to include the three most recent visits. Thus, three visits over 21.8 ± 1.6 months (range 14.7 – 28.9 months) were included for analysis for these patients. Height and liver size were determined at the most recent visit. In those patients who have become transfusion-dependent, the year prior to initiation of regular transfusions was considered the most recent evaluable year. The following hematological parameters were collected for each patient: total hemoglobin concentration, platelet count, WBC, and nRBC/100 WBC. In addition, we compared liver size, height percentile (determined on Tanner-Whitehouse height standard charts), and number of patients who required the initiation of regular red cell transfusions. All characteristics (excluding number of patients now on regular transfusions and gender distribution) were compared with unpaired Students t-test. The number of patients now on regular transfusions and gender distribution were compared using Fisher’s exact analysis.
## 2.3.2.1. Patient Population

**Figure 2.4. Eligibility of δ-thalassemia intermedia patients for comparison of splenectomised vs. non-splenectomised patients**

<table>
<thead>
<tr>
<th>44 patients with δ-Thalassemia Intermedia</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 splenectomised patients</td>
</tr>
<tr>
<td>2 non-evaluable patients</td>
</tr>
<tr>
<td>1 patient splenectomised within 12 months of study</td>
</tr>
<tr>
<td>19 evaluable patients</td>
</tr>
<tr>
<td>1 patient transfused within 12 months of splenectomy</td>
</tr>
<tr>
<td>23 non-splenectomised patients</td>
</tr>
<tr>
<td>10 non-evaluable patients</td>
</tr>
<tr>
<td>1 patient less than 4 years of age</td>
</tr>
<tr>
<td>13 evaluable patients</td>
</tr>
<tr>
<td>1 patient treated for Hodgkin’s Disease</td>
</tr>
<tr>
<td>8 patients transfused before 4 years of age</td>
</tr>
</tbody>
</table>

Five patients (three splenectomised, two non-splenectomised) began regular transfusions at $15.9 \pm 9.5$ (range $4.8 - 54.0$) years. The mean age of the entire study population was $22.3 \pm 2.7$ (4.2 - 57.1) years. The splenectomised patients were significantly older than the non-splenectomised patients; $28.8 \pm 3.4$ (5.9 - 57.1) years in the splenectomised cohort vs. $12.7 \pm 2.8$ (4.2 - 37.4) years in the non-splenectomised cohort ($P=0.03$). The groups were equally distributed in gender; there were 8 males and 11 females in the splenectomised group compared to 8 males and 5 females in the non-splenectomised group ($P=0.47$).
Table 2.7  Clinical characteristics of patients included in β-thalassemia intermedia splenectomy cohort study (n=32)

<table>
<thead>
<tr>
<th>Splenectomised Cohort</th>
<th>Non-Splenectomised Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Sex</td>
</tr>
<tr>
<td>VC</td>
<td>M</td>
</tr>
<tr>
<td>DC</td>
<td>M</td>
</tr>
<tr>
<td>IC</td>
<td>F</td>
</tr>
<tr>
<td>CD</td>
<td>F</td>
</tr>
<tr>
<td>DH</td>
<td>M</td>
</tr>
<tr>
<td>NK</td>
<td>F</td>
</tr>
<tr>
<td>JK</td>
<td>M</td>
</tr>
<tr>
<td>FL</td>
<td>F</td>
</tr>
<tr>
<td>MM</td>
<td>M</td>
</tr>
<tr>
<td>JN</td>
<td>F</td>
</tr>
<tr>
<td>SO</td>
<td>F</td>
</tr>
<tr>
<td>JR</td>
<td>M</td>
</tr>
<tr>
<td>FS</td>
<td>F</td>
</tr>
<tr>
<td>NS</td>
<td>F</td>
</tr>
<tr>
<td>WT</td>
<td>F</td>
</tr>
<tr>
<td>GT</td>
<td>F</td>
</tr>
<tr>
<td>RU</td>
<td>F</td>
</tr>
<tr>
<td>TW</td>
<td>M</td>
</tr>
<tr>
<td>MY</td>
<td>M</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
</tr>
</tbody>
</table>

2.3.3. Results

Comparison of the splenectomised cohort and the non-splenectomised cohort can be found as table 2.8.

Table 2.8  Comparison of splenectomised (n=19) vs. non-splenectomised (n=13) β-thalassemia intermedia patients

<table>
<thead>
<tr>
<th>Splenectomised</th>
<th>Non-Splenectomised</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Male: Female</td>
<td>8:11</td>
<td>8:5</td>
</tr>
<tr>
<td>Current Age</td>
<td>28.8 ± 3.4</td>
<td>12.7 ± 2.8</td>
</tr>
<tr>
<td>Age at Splenectomy</td>
<td>7.8 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Number on Regular Transfusions</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Age at Start</td>
<td>22.1 ± 15.9</td>
<td>6.7 ± 2.7</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>8.0 ± 0.3</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>Platelets (x10^4/L)</td>
<td>688.1 ± 46.9</td>
<td>301.6 ± 28.4</td>
</tr>
<tr>
<td>WBC (x10^3/L)</td>
<td>15.5 ± 0.9</td>
<td>10.7 ± 1.1</td>
</tr>
<tr>
<td>nRBC/100 WBC</td>
<td>401.5 ± 73.9</td>
<td>8.1 ± 3.0</td>
</tr>
<tr>
<td>Height (%ile)</td>
<td>49.9 ± 8.6</td>
<td>33.2 ± 7.1</td>
</tr>
</tbody>
</table>
2.3.3. Comments on comparison of splenectomised vs. non-splenectomised patients with β-Thalassemia Intermedia

In this cohort comparison, the removal of the spleen did not appear to increase basal hemoglobin concentration. However, β-thalassemia intermedia encompasses a broad spectrum of clinical conditions, and it is possible that the splenectomised cohort represented the more clinically severe end of the spectrum. If this were the case, one would expect the splenectomised cohort to have a significantly lower basal hemoglobin concentration than the non-splenectomised cohort, and the fact that the two cohorts had similar hemoglobin concentrations may indicate that splenectomy did improve hemoglobin concentration. Although we do not have documented indications for splenectomy for patients splenectomised in outside centers, patients splenectomised in Toronto displayed falling hemoglobin concentrations and symptomatic anemia, strengthening the theory that the splenectomised cohort may represent the more severe end of the β-thalassemia intermedia spectrum.

As reported by others (Hirsh 1966, Modell 1984), the splenectomised cohort had significantly higher platelets, WBC, and nRBC/100 WBC when compared to the non-splenectomised cohort. However, although pulmonary hypertension has been reported in asplenic anemic patients, in the presence of prolonged thrombocytopenia (Hirsh 1966, Hoeper 1999), no patient in either cohort experienced clinical symptoms of pulmonary hypertension. Effective comparison of pulmonary pressure between the two cohorts, however, is not possible, as neither ECHO cardiograms nor ventricular catheterisation studies have been conducted on any of the patients.

Splenectomy did not affect the number of patients who eventually required the initiation of regular red cell transfusions, nor the age at which these transfusions began. Three of the splenectomised patients and two of the non-splenectomised patients are now on regular transfusions (P>0.99). The splenectomised patients began transfusions at a mean age of 22.1 ± 15.9 (6.1 - 54.3) years, while the non-splenectomised patients began transfusions at 6.7 ± 1.9 (4.8 - 8.6) years (P=0.51).

Post-splenectomy sepsis is a concern, particularly in patients splenectomised as infants. The mean age at splenectomy was about 7 years, with no patient being splenectomised at less than two years of age. In this age group (splenectomised at greater than 2 years old), the reported incidence of infections is almost 11% (Broberger 1960), but we did not see severe infections in either cohort. However, only the most recent twelve months were examined and this was at
least five years after splenectomy in most patients (except for one patient in which it was one year post-splenectomy). As most severe infections are reported within the first twelve to 24 months post-splenectomy (Singer 1973), our analysis may have missed infections that occurred earlier. Also, with only 19 patients in our splenectomised cohort, even an incidence of 11% would yield less than two infections, so a significantly higher incidence of infections in the splenectomised patients may have been missed by the scope of our study.

Although the splenectomised patients in our study did exhibit higher levels of WBC, nRBC, and platelets, there were no significant clinical differences between those patients that were splenectomised and those that were not.
Chapter 3

Splenectomy in Hb E/β-Thalassemia

3.1. Background
As stated in the background to the previous chapter, the ability of splenectomy to increase basal hemoglobin levels is not well studied. In Hb E/β-thalassemia, the use of splenectomy even less clearly defined. The clinical picture of Hb E/β-thalassemia is variable, with some patients requiring regular transfusions, but most surviving without the implementation of regular transfusions. In this population, where many patients are able to survive without regular transfusions, splenectomy may provide an important improvement in quality of life.

3.2. Analysis Pre- and Post-Splenectomy

3.2.1. Purpose of Study
A retrospective study on the effects of splenectomy in patients with Hb E/β-thalassemia was conducted by comparing individual patients pre- and post-splenectomy. The purposes of this study were: (1) to evaluate the impact of splenectomy on hemoglobin concentration; (2) to evaluate the impact of splenectomy on height velocity in patients splenectomised as children; and (3) to determine which baseline parameters, if any, predict response.

We hypothesized that splenectomy would improve hemoglobin concentration in all patients and height velocity in children. We further hypothesized that baseline parameters, including spleen size, age, and baseline hemoglobin concentration would help to predict response.

3.2.2. Experimental Design
The charts of all patients treated at the Kurunegala Teaching Hospital were examined to locate Hb E/β-thalassemia patients who had been splenectomised. In order to be considered eligible for this study, patients needed to be transfusion-independent at the time of splenectomy, and to have pre- and post-splenectomy, non-transfused hemoglobin values determined by coulter. Hemoglobin concentrations were considered transfusion free if they were obtained at least three months after a transfusion. All pre-splenectomy hemoglobin concentrations were meaned to determine a pre-splenectomy value; and, all post-splenectomy hemoglobin concentrations were meaned to determine a post-splenectomy value. Response was defined as an increase in hemoglobin concentration of at least 1.5 g/dL. Height velocities were obtained during the last
year of regular transfusions, the last year of transfusion-independence pre-splenectomy, and the most recent year. Height velocity percentiles were determined using the Tanner-Whitehouse standard charts.

3.2.2.1. Patient Population

Figure 3.1. Eligibility of Hb E/β-thalassemia patients for study pre- and post-splenectomy

106 patients with Hb E/β-Thalassemia have been seen at the Kurunegala Teaching Hospital. Of these, 49 have been splenectomised and fifteen of these patients were evaluable in this study. Of the 34 that were not evaluable, 30 were splenectomised while transfusion-dependent and four do not have any pre-splenectomy hemoglobin concentrations determined by coulter. This is shown in graphic form in figure 3.1.

As shown in table 3.1, the 15 eligible patients included five females and ten males. The mean age at splenectomy was $15.2 \pm 3.1$ (2.5 – 49.8) years, with a mean post-splenectomy follow-up of $1.4 \pm 0.2$ (0.3 – 2.7) years. Current age is $16.9 \pm 3.1$ (3.5 – 50.9) years. Thirteen of the
patients received regular transfusions prior to splenectomy. These transfusions were stopped 27.6 ± 7.6 (6.0 – 113.6) months before splenectomy. The mean spleen size at splenectomy was 16.0 ± 0.7 (11.0 – 20.0) cm. Mean hemoglobin concentration was 4.2 ± 0.2 (3.0 – 5.7) g/dL at baseline and platelet concentration was 384.8 ± 27.3 (205.1 – 613.0) x10^9/L.

Table 3.1 Clinical characteristics of Hb E/β-thalassemia patients analyzed pre- and post-splenectomy (n=15)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at splenectomy (yrs)</th>
<th>Previous Transfusions?</th>
<th>Spleen Size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL 53</td>
<td>F</td>
<td>2.5</td>
<td>Y</td>
<td>16.0</td>
</tr>
<tr>
<td>K 260</td>
<td>M</td>
<td>4.5</td>
<td>Y</td>
<td>20.0</td>
</tr>
<tr>
<td>K 514</td>
<td>M</td>
<td>6.9</td>
<td>N</td>
<td>12.0</td>
</tr>
<tr>
<td>SL 113</td>
<td>M</td>
<td>7.1</td>
<td>Y</td>
<td>11.0</td>
</tr>
<tr>
<td>SL 114</td>
<td>M</td>
<td>8.2</td>
<td>Y</td>
<td>19.0</td>
</tr>
<tr>
<td>SL 168</td>
<td>M</td>
<td>9.4</td>
<td>Y</td>
<td>15.0</td>
</tr>
<tr>
<td>SL 202</td>
<td>F</td>
<td>10.6</td>
<td>Y</td>
<td>19.0</td>
</tr>
<tr>
<td>SL 42</td>
<td>M</td>
<td>11.0</td>
<td>Y</td>
<td>17.0</td>
</tr>
<tr>
<td>SL 27</td>
<td>M</td>
<td>12.8</td>
<td>Y</td>
<td>19.0</td>
</tr>
<tr>
<td>SL 5</td>
<td>M</td>
<td>13.3</td>
<td>Y</td>
<td>18.0</td>
</tr>
<tr>
<td>SL 14</td>
<td>M</td>
<td>13.4</td>
<td>Y</td>
<td>18.0</td>
</tr>
<tr>
<td>SL 100</td>
<td>F</td>
<td>24.0</td>
<td>Y</td>
<td>12.0</td>
</tr>
<tr>
<td>SL 198</td>
<td>F</td>
<td>24.6</td>
<td>N</td>
<td>14.0</td>
</tr>
<tr>
<td>SL 82</td>
<td>F</td>
<td>28.5</td>
<td>Y</td>
<td>15.5</td>
</tr>
<tr>
<td>K 512</td>
<td>M</td>
<td>49.8</td>
<td>Y</td>
<td>15.0</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>15.2</td>
<td></td>
<td>16.0</td>
</tr>
</tbody>
</table>

3.2.3. Hematological Changes Following Splenectomy

Ten of the 15 patients showed a response, defined as an increase in hemoglobin concentration of 1.5 g/dL or greater over that determined at baseline. The responders showed a mean increase of 2.5 ± 0.2 (1.6 – 3.9) g/dL over 1.1 ± 0.3 (0.3 – 2.7) months. The mean change in the five other patients was 0.8 ± 0.3 (-0.3 – 1.4) g/dL over 1.9 ± 0.3 (0.8 – 2.6) months. Individual hemoglobin changes are in table 3.2 for the ten responders and table 3.3 for the five others. Changes are depicted graphically in figure 3.2 with the responders in blue and the non-responders in red. The yellow bar a 1.5 g/dL of hemoglobin represents the threshold of response. All patients remain transfusion-independent.
Table 3.2  Hb concentration at baseline and post-splenectomy for Hb E/β-thalassemia patients with an increase in Hb ≥ 1.5 g/dL following splenectomy (n=10)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-Splenectomy Hb (g/dL)</th>
<th>Post-Splenectomy Hb (g/dL)</th>
<th>Change in Hb (g/dL)</th>
<th>Follow-up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 512</td>
<td>3.3</td>
<td>5.7</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>SL 100</td>
<td>3.4</td>
<td>5.7</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>K 260</td>
<td>3.7</td>
<td>6.9</td>
<td>3.2</td>
<td>0.9</td>
</tr>
<tr>
<td>SL 53</td>
<td>3.9</td>
<td>5.6</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>SL 168</td>
<td>3.9</td>
<td>7.7</td>
<td>3.8</td>
<td>0.3</td>
</tr>
<tr>
<td>SL 27</td>
<td>3.9</td>
<td>6.2</td>
<td>2.3</td>
<td>0.9</td>
</tr>
<tr>
<td>SL 198</td>
<td>4.5</td>
<td>7.2</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>SL 18</td>
<td>4.6</td>
<td>7.0</td>
<td>2.4</td>
<td>0.9</td>
</tr>
<tr>
<td>SL 5</td>
<td>5.0</td>
<td>6.5</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>SL 82</td>
<td>5.7</td>
<td>8.1</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>MEAN</td>
<td>4.1</td>
<td>6.6</td>
<td>2.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 3.3  Hb concentration at baseline and post-splenectomy for Hb E/β-thalassemia patients with an increase in Hb < 1.5 g/dL following splenectomy (n=5)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-Splenectomy Hb (g/dL)</th>
<th>Post-Splenectomy Hb (g/dL)</th>
<th>Change in Hb (g/dL)</th>
<th>Follow-up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 514</td>
<td>3.0</td>
<td>4.4</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>SL 114</td>
<td>3.7</td>
<td>5.1</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>SL 113</td>
<td>3.8</td>
<td>4.3</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>SL 42</td>
<td>4.7</td>
<td>5.4</td>
<td>0.7</td>
<td>2.5</td>
</tr>
<tr>
<td>SL 5</td>
<td>5.5</td>
<td>5.2</td>
<td>-0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>MEAN</td>
<td>4.1</td>
<td>4.9</td>
<td>0.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>
As in the β-thalassemia intermedia patients, there were no significant baseline differences between the ten patients who responded and the five others (see table 3.4). The responders were 13.9 ± 2.9 (2.5 – 28.5) years at splenectomy compared to an age at splenectomy of 17.9 ± 8.0 (7.1 – 49.8) years in the others (P=0.57). One patient in each group had not been on regular transfusions prior to splenectomy (P>0.99). Five of the ten responders were male, as were all five of the other patients (P=0.10). Pre-splenectomy hemoglobin concentration was 4.2 ± 0.2 (3.3 – 5.7) g/dL in the responders and 4.1 ± 0.4 (3.0 – 5.5) g/dL in the others; P=0.93. The responders had shorter follow-up periods, but this did not achieve significance. The follow-up in responders was 1.1 ± 0.3 (0.3 – 2.7) years whereas the others had a follow-up of 1.9 ± 0.3 (0.8 – 2.6) years; P=0.10.
Table 3.4  Comparison of baseline parameters in Hb E/β-thalassemia patients who responded to splenectomy (n=10) and those who did not respond to splenectomy (n=5)

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Others</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Age at splenectomy (yrs)</td>
<td>13.1 ± 2.7</td>
<td>17.9 ± 8.0</td>
<td>0.48</td>
</tr>
<tr>
<td>Male: Female</td>
<td>6:5</td>
<td>5:0</td>
<td>0.12</td>
</tr>
<tr>
<td>Number on regular transfusions pre-splenectomy</td>
<td>10</td>
<td>4</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Spleen size (cm)</td>
<td>16.4 ± 0.7</td>
<td>15.4 ± 1.6</td>
<td>0.52</td>
</tr>
<tr>
<td>Baseline Hb (g/dL)</td>
<td>4.1 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>0.98</td>
</tr>
<tr>
<td>Baseline platelets (x10^9/L)</td>
<td>372.1 ± 41.8</td>
<td>360.2 ± 37.9</td>
<td>0.86</td>
</tr>
<tr>
<td>Follow-up (yrs)</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>0.08</td>
</tr>
</tbody>
</table>

3.2.2.2. Changes in Linear Growth Following Splenectomy

Eleven of the 15 patients were splenectomised as children (under 18 years of age) and therefore the effect of splenectomy on growth was evaluable. All eleven had previously been on regular red cell transfusions. Height velocity while on transfusions was 97.4 ± 0.9 (90 - 99)%ile. In 1997, prior to splenectomy, all patients were removed from regular transfusions and height velocity fell in all patients to 1.5 ± 0.2 (1 - 3)%ile. Height velocity off of transfusions was significantly lower than the height velocities seen while patients were on transfusions, P<0.001. Following splenectomy, height velocity increased in most patients, to 35.7 ± 11.5 (2 - 98)%ile. While this was significantly higher than that seen when patients were off of transfusions (P=0.01), it remained lower than the height velocities observed while patients were transfused (P=0.0004). Individual velocities are included as table 3.5. As shown in figure 3.3, improvement in height velocity was not correlated with hematological improvement; comparison of increase in height velocity to an increase in hemoglobin demonstrates a correlation of $r^2=0.28$; P=0.09.
Table 3.5  Change in height velocity (%ile) post-splenectomy in Hb E/β-thalassemia patients splenectomised as children (n=11)

<table>
<thead>
<tr>
<th>Patient</th>
<th>On transfusions</th>
<th>Off transfusions</th>
<th>Post Splenectomy</th>
<th>Change after Splenectomy</th>
<th>Change in Hb (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL 5</td>
<td>99</td>
<td>1</td>
<td>50</td>
<td>49</td>
<td>-0.3</td>
</tr>
<tr>
<td>SL 113</td>
<td>99</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>SL 42</td>
<td>99</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>SL 114</td>
<td>99</td>
<td>2</td>
<td>6.5</td>
<td>4.5</td>
<td>1.4</td>
</tr>
<tr>
<td>SL 202</td>
<td>99</td>
<td>2</td>
<td>75</td>
<td>73</td>
<td>1.6</td>
</tr>
<tr>
<td>SL 53</td>
<td>90</td>
<td>3</td>
<td>10</td>
<td>7</td>
<td>1.7</td>
</tr>
<tr>
<td>SL 27</td>
<td>99</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>K 512</td>
<td>97</td>
<td>1</td>
<td>25</td>
<td>24</td>
<td>2.4</td>
</tr>
<tr>
<td>SL 14</td>
<td>99</td>
<td>1</td>
<td>25</td>
<td>24</td>
<td>2.4</td>
</tr>
<tr>
<td>K 260</td>
<td>98</td>
<td>2</td>
<td>98</td>
<td>96</td>
<td>3.2</td>
</tr>
<tr>
<td>SL 168</td>
<td>93.5</td>
<td>1</td>
<td>98</td>
<td>97</td>
<td>3.9</td>
</tr>
<tr>
<td>MEAN</td>
<td>97.4</td>
<td>1.5</td>
<td>35.7</td>
<td>34.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

3.2.3. Comments on analysis pre- and post-splenectomy in patients with Hb E/β-thalassemia
Ten of the 15 patients analyzed showed an increase in hemoglobin of at least 1.5 g/dL; and of the five non-responders, two had increases of 1.4 g/dL.

As observed in the patients with β-thalassemia intermedia, no baseline parameters could be used to predict response to splenectomy. The responders have had a shorter follow-up than the others, 1.1 years compared to 1.9 years. Although this does not achieve significance (P=0.08), it is possible that all patients will exhibit an eventual decrease in hemoglobin
concentration following splenectomy, and that some of the patients considered responders would not be so classified if their follow up were longer.

A growth spurt following splenectomy was seen, to varying extents, in seven of eleven patients splenectomised as children. The increase in height velocity was not correlated with the increase seen in hemoglobin concentration ($r^2=0.28; \ P=0.09$). All patients who were splenectomised as children had been on regular transfusions prior to 1997. Growth velocity on transfusions was considerably increased in all patients studied, with all patients achieving velocities above the 90th percentile. Growth velocity off of transfusions demonstrated the severe growth retardation reported by others (Bunn 1986, Logothetis 1972, Modell 1984), with all patients measuring at or below the 3rd percentile. Post-splenectomy, height velocities appeared more normally distributed, resembling what one would expect to find in a given population, with patients ranging from below the 3rd percentile to above the 97th percentile.

Splenectomy in patients with Hb E/β-thalassemia followed in Sri Lanka provided a significant increase in hemoglobin concentration in ten of the 15 patients we analyzed and increased height velocity in seven of eleven patients splenectomised as children. However, there was no baseline parameter that correlated to response, making the decision about which patients to splenectomise difficult.

3.3. Comparison of Splenectomised and Non-Splenectomised Hb E/β-Thalassemia Patients

3.3.1. Purpose of Study

The effect of splenectomy on patients with Hb E/β-thalassemia was also studied by comparing the splenectomised patients with the non-splenectomised patients. The purpose of this study was to compare a cohort of splenectomised patients with a cohort of non-splenectomised patients to determine the effects of splenectomy on: (1) hematological parameters; (2) clinical characteristics; (3) growth; and (4) pulmonary arterial pressure.

3.3.2. Experimental Design

The charts of all patients treated at the Kurunegala Teaching Hospital in Sri Lanka were reviewed, to locate all patients with Hb E/β-Thalassemia. These patients were divided into a splenectomised cohort and a non-splenectomised cohort. These two cohorts were compared in
terms of age at presentation; need to initiate regular transfusions, Hb F, spleen size (pre-splenectomy in the splenectomised cohort), liver size, height percentile, and pulmonary arterial pressure. All comparisons (excluding number previously on regular transfusions and number with pulmonary hypertension) were done by unpaired Student’s T-tests. Number previously on regular transfusions and number with pulmonary hypertension were compared with Fisher’s exact analysis.

3.3.2.1. Patient Population
There were 106 patients with Hb E/β-Thalassemia who were treated at the Kurunegala Teaching Hospital. Of these, 49 were splenectomised and 57 were not splenectomised. Age of the entire population was $18.4 \pm 1.1$ (4.0 – 52.0) years, with splenectomised patients being $20.2 \pm 1.5$ (6.6 – 52.0) years of age and non-splenectomised patients being $16.9 \pm 1.6$ (4.0 – 50.9) years of age. The two cohorts were not significantly different in age, $P=0.14$. The mean age at splenectomy was $13.2 \pm 1.4$ (1.9 – 46.7) years, a mean of $6.7 \pm 0.8$ (0.5 – 31.4) years ago. The gender distribution in the two cohorts was similar; there were 27 males and 22 females in the splenectomised cohort vs. 33 males and 26 females in the non-splenectomised cohort $(P>0.99)$.

This was a retrospective analysis. Therefore, clinical studies had not been performed with this comparison in mind, and all values were obtained through chart review. Not all parameters are collected routinely in clinic, and therefore, not all parameters were available for all patients. Hemoglobin concentrations, determined by coulter, were available over at least 12 months in 80 patients (39 splenectomised and 41 non splenectomised). Therefore, comparison of hematological parameters was done between these 80 patients only. The other parameters were evaluable in patients as indicated in table 3.6.
Table 3.6 Number of Hb E/β-thalassemia patients evaluable for each parameter analyzed in splenectomy cohort study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of Evaluable Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Splenectomised</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
</tr>
<tr>
<td>Current Age</td>
<td>49</td>
</tr>
<tr>
<td>Hemoglobin Concentration</td>
<td>39</td>
</tr>
<tr>
<td>Platelet Concentration</td>
<td>39</td>
</tr>
<tr>
<td>Initiation of Regular Transfusions</td>
<td>49</td>
</tr>
<tr>
<td>Age at Presentation</td>
<td>49</td>
</tr>
<tr>
<td>Fetal Hemoglobin Concentration</td>
<td>49</td>
</tr>
<tr>
<td>Pulmonary Arterial Pressure</td>
<td>40</td>
</tr>
<tr>
<td>Ejection Fraction</td>
<td>45</td>
</tr>
<tr>
<td>Height Percentile</td>
<td>49</td>
</tr>
<tr>
<td>Spleen Size</td>
<td>17</td>
</tr>
<tr>
<td>Liver Size</td>
<td>49</td>
</tr>
</tbody>
</table>

3.3.3. Results

Comparison of the two cohorts is shown as table 3.7; the splenectomised cohort was compared to the non-splenectomised cohort in terms of hematological parameters and pulmonary hypertension.

Table 3.7 Comparison of splenectomised (n=49) and non-splenectomised (n=57) patients

<table>
<thead>
<tr>
<th></th>
<th>Splenectomised</th>
<th>Non-Splenectomised</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>49</td>
<td>57</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Male: Female</td>
<td>27:22</td>
<td>31:26</td>
<td></td>
</tr>
<tr>
<td>Current age (yrs)</td>
<td>20.2 ± 1.5</td>
<td>16.9 ± 1.6</td>
<td>0.14</td>
</tr>
<tr>
<td>Age at splenectomy (yrs)</td>
<td>13.2 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>5.6 ± 0.1</td>
<td>5.0 ± 0.2</td>
<td>0.008</td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td>645.3 ± 33.6</td>
<td>390.6 ± 19.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Height (%ile)</td>
<td>9.9 ± 2.3</td>
<td>11.3 ± 2.1</td>
<td>0.65</td>
</tr>
<tr>
<td>Spleen size (cm)</td>
<td>14.8 ± 0.9</td>
<td>9.7 ± 0.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Liver size (cm)</td>
<td>8.6 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PAP (mmHg)</td>
<td>23.3 ± 1.1</td>
<td>20.3 ± 0.9</td>
<td>0.04</td>
</tr>
<tr>
<td># of patients with PAP ≥ 30 mmHg</td>
<td>8/40</td>
<td>4/46</td>
<td>0.21</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>63.9 ± 1.0</td>
<td>61.8 ± 1.0</td>
<td>0.15</td>
</tr>
</tbody>
</table>

3.3.3.1. Comparison of Hematological Parameters

Eighty patients (39 splenectomised and 41 non-splenectomised patients) were available for hematological comparisons. In these patients, the splenectomised cohort had a significantly higher hemoglobin concentration; 5.6 ± 0.1 (4.0 – 7.7) g/dL in the splenectomised cohort vs. 5.0
± 0.2 (2.6 – 7.6) g/dL in the non-splenectomised cohort; P=0.008. The splenectomised patients also had a higher platelet concentration, 645.3 ± 33.6 (231.5 – 1155.5) x10^9/L vs. 360.6 ± 19.4 (143.5 – 670.3) x10^9/L in the non-splenectomised patients (P<0.0001).

3.3.3.2. Comparison of Pulmonary Arterial Pressure
Eighty-six patients (40 splenectomised and 46 non-splenectomised) had evaluable right heart studies. The mean PAP in the splenectomised cohort was 23.3 ± 1.1 (12 – 37) mmHg compared to a mean of 20.3 ± 0.9 (12 – 40) mmHg in the non-splenectomised cohort (P=0.04). Patients were considered to exhibit pulmonary hypertension if the PAP was equal to or greater than 30 mmHg. Based on this criterion, eight of the 40 splenectomised patients and four of the 46 non-splenectomised patients were hypertensive (P=0.21).

Forty-five splenectomised and 52 non-splenectomised patients had documented cardiac ejection fractions. The ejection fraction in the splenectomised patients was 63.9 ± 1.0 (52 – 80)% . This was not statistically different than the 61.8 ± 1.0 (47 – 79)% seen in the non-splenectomised patients (P=0.15).

3.3.4. Comments on comparison of splenectomised vs. non-splenectomised patients with Hb E/β-thalassemia
As observed by Logothetis (Logothetis 1972), the splenectomised cohort had a greater retardation of linear growth than the non-splenectomised cohort, although this did not achieve significance. As noted by Logothetis, this is likely due to the fact that the splenectomised cohort represented a more severely affected cohort and the increase in linear growth velocity following splenectomy, which has been reported by Modell and others (Blendis 1974, Engelhard 1975), and indeed seen in those patients included in the before and after study, is not enough to allow these patients to catch up to the non-splenectomised cohort. The fact that a significantly larger proportion of splenectomised patients had been on regular transfusions (44 of 49) than seen in the non-splenectomised patients (33 of 57), P=0.004, supports the hypothesis that the patients in the splenectomised cohort were more severely affected. However, further support for this hypothesis, in terms of an earlier age at presentation in the splenectomised group was not observed. The age at presentation in the splenectomised cohort was 7.0 ± 1.2 (0.4 – 38) years, while the non-splenectomised patients presented at a mean age of 8.7 ± 1.3 (0 – 48) years; P=0.33.
Although there was not significantly more splenectomised patients displaying pulmonary hypertension than non-splenectomised patients, there was a trend in that direction, with twice as many splenectomised patients displaying pulmonary hypertension. In addition the splenectomised cohort had a significantly higher mean PAP than was seen in the non-splenectomised cohort (23.3 ± 1.1 mmHg in the splenectomised cohort vs. 20.3 ± 0.9 mmHg in the non-splenectomised cohort; P=0.04).

As opposed to the cohort study in β-thalassemia intermedia patients, the splenectomised cohort had a significantly higher hemoglobin concentration (5.6 ± 0.1 g/dL) when compared to the non-splenectomised cohort (5.0 ± 0.2 g/dL), P=0.008. However, a difference of 0.6 g/dL in total hemoglobin concentration may not represent a clinically significant difference.
The Pharmaceutical Augmentation of Fetal Hemoglobin in Patients with β-Thalassemia

4.1 Treatment with combination of SPB and HU
The purposes of this study were: (1) to evaluate the impact of treatment with SPB, alone and in combination with HU, on hemoglobin concentration in patients with β-thalassemia; (2) to evaluate the relationship between genotype and response to treatment; (3) to determine which baseline parameters, if any, predict response; and (4) to compare red cell survival pre- and post-treatment.

4.1.1. Purpose of Study
We hypothesized that treatment with SPB, in combination with HU, would increase total hemoglobin concentration by augmenting Hb F synthesis and that the greatest response would be seen in patients with genotypes associated with higher levels of Hb F and patients who were younger at baseline. We further hypothesized that ineffective erythropoiesis, as measured by EPO concentration, STfR concentration, bilirubin concentration, and nRBC, would be reduced in those patients exhibiting response.

4.1.2. Patient Population

4.1.2.1. β-Globin Mutation Genotype Grouping
Patients were approached to enroll in the study if they belonged to one of the following genotype groups:
Group 1: Homozygosity or compound heterozygosity for deletions of specific regulatory sequences in the β-globin gene clusters. Examples include δβ-thalassemia and Hb Lepore.
Group 2: Homozygosity or compound heterozygosity for mutations associated with high Hb F production. These are the promoter mutations, including –92, and –88.
Group 3: Homozygosity or compound heterozygosity for mutations unassociated with high Hb F production. These include the mild splicing mutations, including IVS-1 #6.
4.1.2.2. Inclusion/Exclusion Criteria

INCLUSION CRITERIA
Patients also had to meet the following eligibility criteria:

1. Age ≥ 1 year;
2. Transfusion-free for at least 90 days prior to initiation of therapy. Total hemoglobin concentration less than 7.5 g/dL;
3. Folate replete as measured by both serum and red blood cell folate concentrations;
4. Judged by the investigator as unlikely to require transfusion during the study;
5. Willingness to sign informed consent and to comply with study protocol;
6. If sexually active, willing to use regular effective contraception;
7. Absolute neutrophil count ≥ 2.0 x 10^9/L and platelet count ≥ 150,000 x 10^9/L on at least 5 steady-state determinations prior to initiation of therapy;
8. Normal hepatic and renal function (PT ≤ 13 sec; albumin and creatinine within normal limits).

EXCLUSION CRITERIA
Patients were not included in the study if:

1. Breast feeding, pregnant, or planning to become pregnant in next 24 months;
2. On medications associated with neutropenia or thrombocytopenia;
3. Symptoms, signs, or laboratory findings of cardiac disease precluding a potential decline in total hemoglobin concentration before a therapeutic response may be observed.

Twenty-two patients met these criteria, and were enrolled in the study; their baseline characteristics can be found in table 4.1.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype Group</th>
<th>Age</th>
<th>Gender</th>
<th>Transfused?</th>
<th>Hb (g/dL)</th>
<th>Hb F (%)</th>
<th>Splenectomy?</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>3</td>
<td>1.31</td>
<td>M</td>
<td>N</td>
<td>6.1</td>
<td>60.3</td>
<td>N</td>
</tr>
<tr>
<td>SB</td>
<td>3</td>
<td>2.08</td>
<td>F</td>
<td>N</td>
<td>6.0</td>
<td>59.3</td>
<td>N</td>
</tr>
<tr>
<td>JB</td>
<td>3</td>
<td>2.08</td>
<td>F</td>
<td>N</td>
<td>8.6</td>
<td>65.6</td>
<td>N</td>
</tr>
<tr>
<td>GK</td>
<td>3</td>
<td>3.45</td>
<td>M</td>
<td>N</td>
<td>9.0</td>
<td>92.1</td>
<td>N</td>
</tr>
<tr>
<td>SJ</td>
<td>3</td>
<td>3.97</td>
<td>F</td>
<td>N</td>
<td>5.9</td>
<td>92.3</td>
<td>N</td>
</tr>
<tr>
<td>OG</td>
<td>2</td>
<td>4.25</td>
<td>M</td>
<td>N</td>
<td>6.0</td>
<td>72.1</td>
<td>N</td>
</tr>
<tr>
<td>KK</td>
<td>3</td>
<td>5.00</td>
<td>M</td>
<td>N</td>
<td>7.2</td>
<td>92.6</td>
<td>N</td>
</tr>
<tr>
<td>NK</td>
<td>1</td>
<td>5.55</td>
<td>F</td>
<td>N</td>
<td>6.4</td>
<td>78.6</td>
<td>Y</td>
</tr>
<tr>
<td>RD</td>
<td>2</td>
<td>9.02</td>
<td>F</td>
<td>N</td>
<td>6.8</td>
<td>65.5</td>
<td>N</td>
</tr>
<tr>
<td>IC</td>
<td>3</td>
<td>11.74</td>
<td>F</td>
<td>Y</td>
<td>5.9</td>
<td>14.8</td>
<td>Y</td>
</tr>
<tr>
<td>NF</td>
<td>3</td>
<td>17.23</td>
<td>F</td>
<td>Y</td>
<td>6.3</td>
<td>54.4</td>
<td>Y</td>
</tr>
<tr>
<td>TC</td>
<td>3</td>
<td>19.43</td>
<td>F</td>
<td>Y</td>
<td>7.1</td>
<td>44.4</td>
<td>Y</td>
</tr>
<tr>
<td>AT</td>
<td>3</td>
<td>21.29</td>
<td>M</td>
<td>Y</td>
<td>5.4</td>
<td>31.3</td>
<td>N</td>
</tr>
<tr>
<td>BE</td>
<td>3</td>
<td>25.68</td>
<td>F</td>
<td>Y</td>
<td>6.7</td>
<td>13.0</td>
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<tr>
<td>AC</td>
<td>3</td>
<td>29.61</td>
<td>F</td>
<td>Y</td>
<td>7.4</td>
<td>50.9</td>
<td>Y</td>
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<td>NB</td>
<td>3</td>
<td>32.70</td>
<td>F</td>
<td>Y</td>
<td>7.0</td>
<td>47.9</td>
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<tr>
<td>CZ-1</td>
<td>3</td>
<td>34.52</td>
<td>M</td>
<td>N</td>
<td>5.0</td>
<td>82.6</td>
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<td>CZ-2</td>
<td>3</td>
<td>36.06</td>
<td>M</td>
<td>N</td>
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<td>85.6</td>
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<td>38.31*</td>
<td>M</td>
<td>Y</td>
<td>n/a</td>
<td>n/a</td>
<td>Y</td>
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<tr>
<td>SA</td>
<td>3</td>
<td>12.64*</td>
<td>F</td>
<td>Y</td>
<td>n/a</td>
<td>n/a</td>
<td>N</td>
</tr>
<tr>
<td>MR</td>
<td>3</td>
<td>12.52*</td>
<td>M</td>
<td>Y</td>
<td>n/a</td>
<td>n/a</td>
<td>N</td>
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<td>LT</td>
<td>3</td>
<td>23.30*</td>
<td>F</td>
<td>Y</td>
<td>n/a</td>
<td>n/a</td>
<td>Y</td>
</tr>
</tbody>
</table>

* For patients who dropped out before baseline, current age is given.
4.1.3. Experimental Protocol

A schematic of the protocol can be seen as figure 4.1. Patients received folic acid during all phases of therapy.

**Figure 4.1 Pharmacological protocol for SPB and HU study**

- **3 Months** Run-in Phase
- **3 Months** SPB
- **3 Months** SPB and HU
- **6 Months** HU

**Phase 1**
Duration: 3 months
Patients were treated with SPB at 12 g/m²/day in three divided doses. Adult patients took this in capsule form and children received a powered form that was dissolved in water, juice, or pudding.

**Phase 2**
Duration: 3 months
HU, administered by mouth was added to SPB, at a starting dose of 7 mg/kg body weight once daily. This daily dose was increased by 2.5 mg/kg every eight to twelve weeks, if no toxicity was observed, to maximum tolerated dose, which was determined to be reached if hematological toxicity was noted (platelet count below 150,000 x 10⁹/L or absolute neutrophil count below 2.0 x 10⁹/L). HU was discontinued until return of blood counts to normal. After recovery, HU was resumed at a dose 2.5 mg/kg below that which caused toxicity; this became the patient’s maximum tolerated dose. Dose adjustment was based upon the patient’s weight at the time that the adjustment was due.

**Phase 3**
Duration: 6 months
SPB was discontinued, and HU was given as a single agent.

4.1.3.1. Clinic Visits

Baseline
Clinical

1. Medical History
2. Physical examination, including weight, height, and Tanner Stage

Laboratory
See Table 4.2

Follow-up
Clinical

Physical examination, including weight, height, and Tanner Stage

Laboratory
See Table 4.2
### Table 4.2 SPB and HU study protocol table

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-Baseline</th>
<th>Baseline</th>
<th>Treatment Phase 1 3 months SPB alone</th>
<th>Treatment Phase 2 3 months SPB and HU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks from baseline</td>
<td>-4</td>
<td>0</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Serum and RBC folate</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CBC, differential</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Retic</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>NRBC</td>
<td>X</td>
<td>X</td>
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<td>Plasma Hb</td>
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<tr>
<td>Hb F (Toronto)</td>
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<td>X</td>
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<td>X</td>
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<td>Hb F (Oxford)</td>
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<td>X</td>
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<td>Serum Bilirubin</td>
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<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Cr/ALT/PT</td>
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<td>α/non α chain ratio</td>
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<tr>
<td>Red cell survival</td>
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</tbody>
</table>
4.1.4. Results
Twenty-two patients were enrolled in this study. Twelve patients reached the end of treatment phase 2 (SPB and HU). Thirteen patients dropped out of the study (table 4.3) for the following reasons: inability to tolerate low hemoglobin concentration during weaning from transfusions (n=4); inability to tolerate low hemoglobin concentration during treatment in which response was not seen (n=4); inability to tolerate even low doses of HU (n=2); inability to tolerate SPB (n=2); and inability to follow protocol (n=1). Four patients withdrew after six months of treatment, and are included in the twelve patients evaluable. One patient has been on treatment for less than six months and is, therefore, not being included in this analysis. This is shown in figure 4.2.

Figure 4.2  Eligibility of patients enrolled in SPB and HU trial for analysis in this study
Table 4.3 Patients who dropped out of SPB and HU study (n=13)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Months of Therapy at discontinuation</th>
<th>Therapy Received</th>
<th>Reason for discontinuation</th>
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<tr>
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<tr>
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<tr>
<td>TC</td>
<td>F</td>
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<td>0.5</td>
<td>SPB</td>
<td>SPB toxicity</td>
</tr>
<tr>
<td>NB</td>
<td>F</td>
<td>32.70</td>
<td>3</td>
<td>SPB</td>
<td>SPB toxicity</td>
</tr>
<tr>
<td>AC</td>
<td>F</td>
<td>29.61</td>
<td>3</td>
<td>SPB</td>
<td>Not responding</td>
</tr>
<tr>
<td>IC</td>
<td>F</td>
<td>11.74</td>
<td>3</td>
<td>SPB</td>
<td>Not responding</td>
</tr>
<tr>
<td>BE</td>
<td>F</td>
<td>25.68</td>
<td>4</td>
<td>SPB, SPB/HU</td>
<td>Not responding</td>
</tr>
<tr>
<td>CZ-1</td>
<td>M</td>
<td>34.52</td>
<td>7</td>
<td>SPB, SPB/HU</td>
<td>HU toxicity</td>
</tr>
<tr>
<td>SB</td>
<td>F</td>
<td>2.08</td>
<td>10</td>
<td>SPB, SPB/HU, HU</td>
<td>HU toxicity</td>
</tr>
<tr>
<td>OG</td>
<td>M</td>
<td>4.25</td>
<td>10</td>
<td>SPB, SPB/HU, HU</td>
<td>Transfused in India</td>
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<tr>
<td>SG</td>
<td>M</td>
<td>1.31</td>
<td>11</td>
<td>SPB, SPB/HU, HU</td>
<td>Not responding</td>
</tr>
</tbody>
</table>

*For patients who dropped out before baseline, current age is given.

The twelve patients analyzed were 9.1 ± 2.9 (1.3 – 34.5) years at baseline and all were followed at the Hemoglobinopathy Program at The Hospital for Sick Children or the Toronto General Hospital, General Division, Toronto, Canada. Two patients were transfusion-dependent, and were successfully weaned from transfusions before the initiation of therapy.

After three months of therapy with SPB, the mean change in Hb F was from a baseline of 70.6 ± 5.3 (31.3 – 92.6)% to 74.7 ± 3.9 (54.3 – 98.2)%; P=0.17. This represented a change in absolute Hb F from a baseline of 4.7 ± 0.5 (1.7 – 8.3) g/dL to 5.9 ± 0.6 (3.8 – 9.7) g/dL post-treatment (P=0.0003). Over the same period, total hemoglobin concentration increased from a baseline of 6.5 ± 0.3 (5.0 – 9.0) g/dL to 7.5 ± 0.5 (4.1 – 9.9) g/dL (P=0.001). Two patients showed an increase in total hemoglobin of at least 1.5 g/dL (1.8 g/dL and 1.9 g/dL) and were thus considered responders to SPB alone.

Hb F increased from 70.6 ± 5.3 (31.3 – 92.6)% before initiation of therapy to 77.3 ± 4.5 (54.6 – 95.7)% after the second phase therapy (P = 0.01). Mean change in Hb F was 7.85 ± 2.63 (-6.4 – 23.3)%. This represented a change in absolute Hb F of 1.2 ± 0.7 (0.4 – 2.1) g/dL; from a baseline of 4.7 ± 0.5 (1.7 – 8.3) g/dL to 6.4 ± 0.6 (3.4 – 8.9) g/dL after treatment with SPB and HU. Change in total hemoglobin concentration between baseline and the end of the two phases of therapy was from 6.5 ± 0.3 (5.0 – 9.0) g/dL to
7.8 ± 0.5 (4.2 – 10.6) g/dL (P=0.003). The mean change in total hemoglobin concentration was 1.3 ± 3.3 (-0.8 – 3.0) g/dL. The two patients who responded to SPB continued to show a response; while, four patients who had not responded to SPB alone showed responses after the addition of HU. The mean increase in the six patients who showed a response was 2.3 ± 0.2 (1.5 – 3.0) g/dL compared to hemoglobin concentration determined at baseline. Six patients continued to be non-responsive, mean change in total hemoglobin concentration in these six patients was 0.3 ± 0.3 (-0.8 – 1.0) g/dL.

Change in total hemoglobin concentration between baseline, treatment with SPB, and treatment with SPB and HU is depicted in figure 4.3. Figure 4.4 depicts the change in Hb F over the same periods.

Non-significant increases were seen with the addition of HU to SPB. Hb F increased from 74.7 ± 3.9 (54.3 – 98.2)% before HU was added to 77.3 ± 4.5 (54.6 – 95.7)% after three months of combination treatment (P=0.32). Absolute Hb F was 5.9 ± 0.6 (3.8 – 9.7) g/dL after treatment with SPB alone, and increased to 6.4 ± 0.6 (3.4 – 8.9) g/dL after three months of SPB and HU. Total hemoglobin concentration was 7.5 ± 0.5 (4.1 – 9.9) g/dL after three months of treatment with SPB alone and increased to 7.8 ± 0.5 (4.2 – 10.6) g/dL after HU had been added for three months (P=0.08).

Table 4.4  Change in hematological Parameters following treatment with SPB and SPB & HU in 12 patients with severe β-Thalassemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>After SPB</th>
<th>After SPB &amp; HU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>6.5 ± 0.4</td>
<td>7.5 ± 0.5</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td>Hb F (g/dL)</td>
<td>4.7 ± 0.5</td>
<td>5.9 ± 0.6</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>nRBC/100 WBC</td>
<td>125.1 ± 80.0</td>
<td>81.7 ± 54.5</td>
<td>87.9 ± 66.5</td>
</tr>
<tr>
<td>[EPO] (IU/L)</td>
<td>693.0 ± 211.9</td>
<td>487.8 ± 189.3</td>
<td>253.0 ± 59.2</td>
</tr>
<tr>
<td>[STfR] (mg/L)</td>
<td>23.7 ± 3.6</td>
<td>15.5 ± 4.0</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>Bilirubin (μmol/L)</td>
<td>34.7 ± 6.5</td>
<td>19.9 ± 3.6</td>
<td>18.7 ± 2.2</td>
</tr>
</tbody>
</table>
Figure 4.3  Change in total Hb (g/dL) during treatment in patients treated in SPB and HU study for at least six months (n=12)
Serum EPO concentration decreased (although not significantly) from 693.0 ± 211.9 (128-1350) IU/L before initiation of therapy to 487.8 ± 189.3 (47 - 1350) IU/L after three months of SPB (P=0.20), and further decreased to 253.0 ± 59.2 (42- 400) IU/L (P=0.12, compared to baseline) after three months of SPB and HU. Over the same intervals, STfR concentration went from 23.7 ± 3.7 (12 - 38) mg/L to 15.50 ± 4.0 (8 - 35) mg/L (P =0.007) and then to 10.5 ± 1.1 (7 - 15) mg/L (P=0.007, compared to baseline).

Further indicators of reduction in ineffective erythropoiesis included reductions in unconjugated bilirubin and nRBC. Bilirubin was determined at baseline, end of treatment with SPB, and end of treatment with SPB and HU. At baseline, bilirubin was 34.7 ± 6.5 (13 - 89) μmol/L. It was 19.9 ± 3.6 (9 - 47) μmol/L after three months of SPB alone and 18.7 ± 2.2 (10-34) μmol/L after three months of combination treatment. The significance between baseline and SPB therapy, and SPB and HU therapy was P=0.005 and P=0.017, respectively. A decrease in nRBC was also observed, although it did not achieve significance. At baseline, patients had 125.1 ± 80.0 (3 - 873) nRBC/100 WBC. This decreased to 81.7 ± 54.5 (0 - 618) nRBC/100 WBC after three months of treatment.
with SPB (P=0.40, compared to baseline) and to $87.9 \pm 66.5 \ (0-803)$ nRBC/100 WBC after HU had been added for three months (P = 0.58, compared to baseline).

4.1.4. Comments on treatment with SPB and HU

Similar to other reports (Collins 1995, Dover 1998, Faller 1995, Perrine 1993, Sher 1995), we observed variable responses to treatment with SPB in our patients. Of the 12 patients, an increase in total hemoglobin concentration of at least 1.5 g/dL was seen in two patients after three months of treatment with SPB. In one patient, total hemoglobin rose from 6.0 g/dL at baseline to 7.8 g/dL after three months of treatment with SPB. Hb F rose from 72.1% to 98.2%; absolute Hb F rose from 4.3 g/dL to 6.5g/dL. The second patient had a baseline total hemoglobin concentration of 7.2 g/dL, which rose to 9.1 g/dL at the same time that Hb F remained relatively constant, from 92.6% to 93.5%, with absolute Hb F changing from 6.7 g/dL to 8.5 g/dL. Although the two responders had a significantly higher increase in total hemoglobin concentration ($1.9 \pm 0.1 \text{ g/dL}$ in the responders vs. $0.7 \pm 0.7 \text{ g/dL}$ in the others; P=0.04), they did not have a significantly higher increase in Hb F ($5.9 \pm 5.0\%$ in the responders vs. $5.1 \pm 4.2 \%$ in the others; P=0.9) when compared to the others. This lack of correlation between response in total hemoglobin and Hb F may be partially explained by the fact that one of the responders had a baseline Hb F of 92.6%, leaving little room for an increase. Also, one of the non-responders began with an Hb F level of 31.3% and a Hb A of 63.6%; this patient was transfusion-dependent and these values may represent a significant amount of transfused blood. Therefore, the increase seen in Hb F is likely due, in part, to a return to endogenous globin production levels as the transfused blood was removed.

Although Collins reported a correlation between response to SPB and both baseline EPO concentration and baseline Hb F, with patients with higher baseline levels showing better responses (Collins 1995), this was not observed in our twelve patients. All patients had baseline EPO concentrations greater than or equal to 120 IU/ L (which Collins associated with a response); and two responded to the SPB, while ten did not (P > 0.99). Similarly, we did not observe that baseline levels of Hb F predicted response. Collins reported that baseline Hb F greater than or equal to 40% was correlated to response. Both of our responders had over 40% Hb F at baseline, but so did nine of the ten non-responders (P > 0.99).
Both of the patients who had shown a response after three months of treatment with SPB alone continued to show a response when HU was added. Of the ten patients who did not respond to SPB, four showed a response when HU was added, while six remained unresponsive.

There have only been two reports on the use of both HU and SPB to augment Hb F; Olivieri (1998) reported two siblings with Hb Lepore, and Hoppe (1999) reported on two patients with Hb E/β-thalassemia in whom SPB was added to a regimen of HU. Olivieri reported an additive effect between SPB and HU. Hoppe reported an increase in Hb F with the addition of SPB to HU, but saw no increase in total hemoglobin concentration. The mean increase with the addition of HU in all twelve patients was $0.4 \pm 0.2$ (-0.6 - 1.3) g/dL. Of the ten patients who had not shown a response of at least 1.5 g/dL with SPB alone, four showed this response when HU was added to the treatment regimen; the mean increase in total hemoglobin concentration in these four patients was $1.0 \pm 0.2$ (0.5 - 1.3) g/dL.

Based on earlier work, we hypothesized that patients in genotype group 1 (δβ-thalassemia or Hb Lepore) would show a greater response than those in the other genotype groups (Olivieri 1998), and that those in group 3 (homozygosity or heterozygosity for mutations unassociated with high Hb F production) would show little or no response (Dover 1998). However, this was not observed in our patient population. Of the six responders, five were in group 3 and one was in group 2. The non-responders included four patients from group 3, one from group 2, and one from group 1. There were no significant differences in the proportion of responders seen in each group ($P=0.57$). However, the number of patients in each group varied: there was only one patient from group 1 and two from group 2, while there were nine patients from group 3. Therefore, the lack of response seen in groups 1 and 2 may have been an artifact of the small patient populations in these groups. An effort to include more patients from group 1 by enrolling patients from a center in Macedonia was not successful due to logistic problems in collaborating with the Macedonian center. This included the state of war in Macedonia, protocol violations by investigators in Macedonia, and difficulty getting drug supply to Macedonia.
Although our use of SPB and HU in patients with β-thalassemia did not produce an increase in total hemoglobin concentration in all patients, it is worthwhile to try in patients, particularly those that are currently transfusion-independent but may be soon requiring intervention. As seen in the four patients who did not show a significant increase in total hemoglobin concentration during treatment with SPB alone, but did show a significant response after the addition of HU, the use of SPB and HU in combination may provide more effective treatment than SPB alone. The adverse effects seen with the administration of SPB (GI upset, phlebitis, dizziness, and somnolence) or HU (neutropenia) were all alleviated with the cessation of treatment (usually temporary). Thus, a trial of SPB and/or HU, before the initiation of regular transfusions, may be warranted.

4.2. Augmentation of Fetal Hemoglobin in infants
Pace et al. (1994) used three transgenic mouse models to show that butyrates were more effective at augmenting Hb F when endogenous γ-globin production is at high levels. Furthermore, in clinical studies, Collins reported that patients with endogenous Hb F levels greater than 40% were significantly more likely to respond to treatment with SPB than those with endogenous levels below 40% (Collins 1995).

4.2.1. Purpose of Study
The objective of this study was to evaluate the impact of treatment with SPB on total hemoglobin and Hb F in infants. We hypothesized that these patients, in whom the silencing of the γ-globin gene is not yet complete, would respond better to SPB than other reports in the literature, which involved adults and older children.

4.2.2. Patient Population
Five infants, aged 7.0 ± 2.6 (1.6 – 16.0) months, were treated as outpatients for the administration of oral SPB. All patients were homozygous for β-thalassemia, and determinations of β globin mutations were determined by DNA analysis (table 4.5). One patient had received six transfusions and a second had received one transfusion prior to the start of therapy. The other three patients had not previously been transfused.
4.2.4. Results

All patients poorly tolerated drug administration and parental histories revealed refusal and spitting up of feeds, presumably due to the bitterness of the compound. Compliance, however, was reported to be consistently good in all patients. There were no specific adverse events attributable to SPB therapy.

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As shown in table 4.7, overall, the response to therapy was disappointing. Mean hemoglobin prior to therapy was 6.6 ± 0.8 (4.8 – 8.8) g/dL, and showed no significant increase during or following SPB administration; hemoglobin following therapy was 6.1 ± 0.6 (4.3 – 8.0) g/dL, P = 0.53. No individual patient showed an increase equal to or greater than 1.5 g/dL; mean change in Hb was −0.5 ± 0.70 (-2.9 – 1.4) g/dL. Change in Hb F was also not significant. Hb F dropped from 87.5 ± 7.0 (61.7 – 100)% at baseline to 75.3 ± 15.9 (28.9 – 98.7)% at study end (P=0.29); absolute Hb F in changed from 5.9 ± 1.1 (3.2 – 8.8) g/dL to 4.7 ± 1.2 (1.9 – 7.5) g/dL over the same period (P=0.29).

Pre- and post-treatment EPO and STfR concentrations were available for two patients. There was no change in EPO concentration; both patients had values above 500 IU/L at both baseline and study end. Non-significant increases in STfR concentrations were seen in these two patients. One patient went from a baseline value of 11.1 mg/mL to 13.2 mg/mL after treatment; while the second patient increased from a baseline of 8.7 mg/mL to 12.0 mg/mL after treatment. There was a non-significant increase in nRBC/100 WBC in the five patients. At baseline, there was a mean of 30 nRBC/100 WBC, which increased to 36 nRBC/100 WBC at study end (P=0.31).

Table 4.7 Hematological response to treatment with SPB in infants (n=5)

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<th>Hb F (g/dL)</th>
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</thead>
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<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
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<td>4.8</td>
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<td>4.0</td>
</tr>
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<td>5.9</td>
<td>6.3</td>
</tr>
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<td>7.8</td>
<td>8.0</td>
<td>7.5</td>
</tr>
<tr>
<td>ML</td>
<td>8.8</td>
<td>5.9</td>
<td>8.8</td>
</tr>
<tr>
<td>MEAN</td>
<td>6.6</td>
<td>6.1</td>
<td>87.5</td>
</tr>
</tbody>
</table>

4.2.5. Comments of augmentation of Hb F in infants

Published reports on the use of SPB to augment Hb F in patients with β-thalassemia have been varied, but the overall results have been disappointing (Collins 1995, Dover 1998, Ikuta 1998, Olivieri 1998, Perrine 1993, Reich 2000). The use of SPB to treat infants, in whom the silencing of the γ-globin gene is not yet complete, was based on work by Pace et al. (1994) who used three transgenic mouse models to show that butyrate was more effective at augmenting Hb F when the endogenous γ-globin
production was at high levels (Pace 1994). Furthermore, in clinical studies, Collins reported that patients with endogenous Hb F levels greater than 40% were significantly more likely to respond to treatment with SPB than those with endogenous levels below 40% (Collins 1995). However, our use of SPB in five infants proved disappointing, with no patient showing a response to treatment.

Pace et al. (1994) utilized a μLCRAγ cassette in the high γ-globin expression mice and a β locus YAC cassette in the silenced γ-globin mice. In the μLCRAγ mouse, treatment with α-amino butyric acid led to a 3.4- to 6.4-fold increase in γmRNA production, while no increase in γmRNA production was seen in the β locus YAC transgenic mice. The μLCRAγ transgenic mouse construct did not include any competing globin chains, so it may not be paralleled in clinical practice. Our data suggest, in fact, that Pace’s results are not observed in clinical practice.

The discord between our results and those seen by Collins, who proposed that baseline Hb F levels greater than 40% predicted a response, may be explained by the fact that all of the patients in Collins’ analysis were older children or adults, in whom high levels of Hb F represented persistence of γ-globin production whereas our patients were infants in whom the high levels of Hb F were expected.

There was no evidence that treatment with SPB led to a reduction in ineffective erythropoiesis. There was a non-significant overall increase in nRBC/100 WBC. Furthermore, in the two patients in whom it was available, serum EPO concentration remained above 500 IU/L, while STfR concentration increased in both patients.

Although we hypothesized that the augmentation of Hb F would be more effective in infants, in whom we were preventing the switch off of the γ-globin gene, as opposed to trying to re-activate a downregulated gene, none of the five infants that we treated with SPB showed a response. All five patients were begun on regular transfusions at the end of this study.
Chapter 5

Red Cell Survival

5.1 Background
Red cell survival studies were preformed before patients began studies to augment Hb F synthesis with SPB and HU. In vitro biotin labeling of a random sample was utilized in this analysis. The objective of this study was to determine baseline red cell survival, against which, red cell survival post-treatment could be compared.

5.2. Laboratory Methods
Patients were registered at the out patient clinic in the morning, and an intravenous line was started in each arm. Twenty-five to 35 cc of whole blood were removed through one of the intravenous lines and lithium heparin (15 U/mL) was added. The whole blood was centrifuged at 1500 rpm for 5 minutes and the plasma discarded. The red blood cells were washed with phosphate buffered saline (PBS) three times. NHS-biotin was dissolved in sterile water to a concentration of 1 mM. This was added to the packed red blood cells (pRBC) in a 1:40 ratio. PBS was added to adjust the hematocrit to 0.80 (a ratio of 1:4, pRBC to PBS was used). The solution was allowed to incubate for 30 minutes with periodic inversion of the tube. The mixture was then washed three times with PBS and once with sterile saline. The labeled red cells were mixed with an equal volume of sterile saline and re-infused into the patient. The subsequent time points were drawn through the second intravenous line to avoid possible contamination. The time points on day 0 were 5, 7.5, 10, 12.5, 15, 20, and 60 minutes after the end of infusion. Following the 60 minute sample, the intravenous lines were removed and the patient was discharged. Samples were taken at 24, 48, and 72 hours post infusion, and weekly thereafter until the amount of biotin labeled red blood cells became insignificant.

To measure the amount of biotin label in each sample, flow cytometry methods were utilized. The labeled red cells were suspended in 500 μL HBS. The suspension was incubated with 1 mg/mL streptavidin, R-phycoerythrin in 2 mM azide and 275 μL HCS for 30 minutes at room temperature. The mixture was then spun, and the supernatant discarded. The red blood cells were resuspended in 300 μL HBS, and analyzed in FACS.
The mean percent labeled red cells from day 0 were taken to be 100% and the levels obtained on subsequent draws were compared to this value. The lowest level of biotin detected in the samples by FACS was 0.01%. The points were plotted in a linear fashion and the survival curve was taken as the exponential line of best fit.

5.3. Patient Population
Red cell survival studies were done in one control group and two patient populations: patients with β-thalassemia and patients with Hb E/β-thalassemia

5.3.1. Normal Controls
Two normal controls (1 male and 1 female) underwent red cell survival studies. Both patients were adults; neither had been splenectomised.

5.3.2. Patients with β-Thalassemia
Red cell survival studies have been performed on four patients with β-Thalassemia, two females and two males. These patients were all adults; mean age at study start was 23.4 ± 4.3 (17.0 – 35.9) years. Three patients had had splenectomies and three were transfusion-dependent, while one had a 13-year history of transfusion-independence. All transfusion-dependent patients were weaned from transfusions before beginning red cell survival studies. As shown in table 5.1, all patients had β⁺ mutations and the full complement of α genes. In the transfusion-dependent patients, 40.3 ± 10.6 (26 – 61) days elapsed between the final transfusion (5 cc/kg in all cases) and the first day of biotin labeling. Table 5.2 shows the hematological characteristics of these four patients. Levels of Hb A in the three transfusion-dependent patients was 86.8 ± 3.2 (83.6 – 90.6)% when the study was initiated; these levels decreased to 56.9 ± 6.3 (44.4 – 63.6)% by study completion. In the transfusion-independent patient, Hb A was 84.1% at study start and 85.6% at study end. However, total hemoglobin concentration in the four patients did not change; it was 6.6 ± 0.2 (6.1 – 7.0) g/dL at baseline and 6.6 ± 0.5 (5.4 – 7.1) g/dL at study completion.
Table 5.1  Clinical characteristics of β-thalassemia patients enrolled in red cell survival study (n=4)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Genotype</th>
<th>Transfusions?</th>
<th>Splenectomy?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>F</td>
<td>17.02</td>
<td>β⁺/β⁺</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>TC</td>
<td>F</td>
<td>19.3</td>
<td>β⁺/β⁺</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>AT</td>
<td>M</td>
<td>21.2</td>
<td>β⁺/β⁺</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>CZ</td>
<td>M</td>
<td>35.9</td>
<td>β⁺/β⁺</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table 5.2  Hematological parameters of β-thalassemia patients enrolled in red cell survival study (n=4)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Study Start</th>
<th>Study End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Hb (g/dL)</td>
<td>Hb A (%)</td>
</tr>
<tr>
<td>NF</td>
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</tr>
<tr>
<td>TC</td>
<td>6.1</td>
<td>83.6</td>
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<tr>
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<td>n/a</td>
</tr>
<tr>
<td>CZ</td>
<td>6.7</td>
<td>84.1</td>
</tr>
</tbody>
</table>

5.3.3. Patients with Hb E/β-Thalassemia

Red cell survival studies were also performed in seven patients with Hb E/β-thalassemia, three females and four males. Mean age at study start was 17.2 ± 1.0 (12.3 - 19.8) years. One patient had been splenectomised; all seven patients were transfusion-dependent, and were successfully weaned from transfusions before the studies began. As shown in table 5.3, 6 patients had β⁰ mutations and 1 patient had a β⁺ mutation. All patients had the full complement of α genes.

Biotin labeling of red cells was performed 77.4 ± 7.9 (53.3 – 113.1) days after the final transfusion (5 – 10 cc/kg). Levels of Hb A in these seven patients were 22.0 ± 2.9 (12.9 – 33.2)% when the study was initiated and decreased to 16.2 ± 1.0 (14.3 – 17.4)% at study completion. Total hemoglobin concentration was 6.7 ± 0.4 (5.8 – 8.3) g/dL at study start and 5.7 ± 0.1 (5.6 – 5.9) g/dL at study end. Individual hematological values are shown in table 5.4.
Table 5.3  Clinical characteristics of Hb E/β-thalassemia patients enrolled in red cell survival study (n=7)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Genotype</th>
<th>Transfusions?</th>
<th>Splenectomy?</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC</td>
<td>F</td>
<td>12.3</td>
<td>β⁰</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>KC</td>
<td>M</td>
<td>15.3</td>
<td>β⁰</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>TK</td>
<td>M</td>
<td>17.8</td>
<td>β⁰</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>MJ</td>
<td>F</td>
<td>18.0</td>
<td>β⁺</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>OS</td>
<td>F</td>
<td>18.2</td>
<td>β⁰</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>VL</td>
<td>M</td>
<td>19.3</td>
<td>β⁰</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>PS</td>
<td>M</td>
<td>19.8</td>
<td>β⁰</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table 5.4  Hematological parameters of Hb E/β-thalassemia patients enrolled in red cell survival study (n=7)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total Hb (g/dL)</th>
<th>Hb A (%)</th>
<th>Total Hb (g/dL)</th>
<th>Hb A %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ</td>
<td>5.8</td>
<td>29.2</td>
<td>5.9</td>
<td>16.9</td>
</tr>
<tr>
<td>KC</td>
<td>5.9</td>
<td>24.6</td>
<td>5.6</td>
<td>14.3</td>
</tr>
<tr>
<td>JC</td>
<td>6.0</td>
<td>33.2</td>
<td>5.8</td>
<td>17.4</td>
</tr>
<tr>
<td>PS</td>
<td>7.4</td>
<td>12.9</td>
<td>7.1</td>
<td>0%</td>
</tr>
<tr>
<td>OS</td>
<td>6.4</td>
<td>13.6</td>
<td>6.6</td>
<td>0%</td>
</tr>
<tr>
<td>TK</td>
<td>7.3</td>
<td>18.8</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>VL</td>
<td>8.3</td>
<td>21.4</td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

5.4. Results

5.4.1. Normal Controls

Red cell survival studies were performed in two controls. One control underwent two studies, to establish study reliability. A plot of the curves is found as figure 5.1.

The last day of detection for the first control was 84 days post-infusion in both studies. Both studies gave a linear red cell half-life of 42 days. In the second control, last day of biotin detection was 77 days after infusion, and linear half-life was 39 days. This is reported in table 5.5.
5.4.2. Patients with β-Thalassemia

All four patients had reduced red cell survival times, with the final day of biotin detection ranging from 11 to 49 days post-infusion and linear half-lives of 9 to 34 days. A plot of the survival curves is found as figure 5.2.
The amount of biotinylated cells detected on day 0 and at the day that biotinylated cells were last detected are shown in table 5.6. Also shown in this table are the day on which biotinylated cells were last detected for each patient, and the percent of the day 0 value that this represented. Calculated red cell half-life for each patient is also included.

**Table 5.6 Amount of biotin detected at baseline and study completion, and calculated red cell half lives for patients with β-thalassemia (n=4)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>% biotinylated RBC at day 0</th>
<th>% biotinylated RBC at last detection</th>
<th>% of day 0 at last detection</th>
<th>Day of last detection</th>
<th>Linear Half-life (days)</th>
<th>Exponential Half-life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZ</td>
<td>0.26</td>
<td>0.04</td>
<td>15.4</td>
<td>49</td>
<td>33</td>
<td>16</td>
</tr>
<tr>
<td>CT</td>
<td>0.30</td>
<td>0.17</td>
<td>56.5</td>
<td>28</td>
<td>34</td>
<td>14</td>
</tr>
<tr>
<td>AT</td>
<td>0.38</td>
<td>0.01</td>
<td>3.0</td>
<td>11</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>NF</td>
<td>0.56</td>
<td>0.08</td>
<td>13.9</td>
<td>42</td>
<td>24</td>
<td>11</td>
</tr>
</tbody>
</table>

**5.4.3. Patients with Hb E/β-Thalassemia**

All patients showed a reduced red cell survival, with the final day of biotin detection ranging from 14 to 42 days and linear red cell half-lives of 9 to 28 days. The survival curves are depicted in figure 5.3.
The amount of biotinylated cells detected on day 0 and at the day that biotinylated cells were last detected are shown in Table 5.7. Also shown in this table are the day on which biotinylated cells were last detected for each patient, and the percent of the day 0 value that this represented. Calculated red cell half-life for each patient is also included.

Table 5.7 Amount of biotin detected at baseline and study completion, and calculated red cell half lives for patients with Hb E/β-thalassemia (n=7)

<table>
<thead>
<tr>
<th>Patient</th>
<th>% biotinylated RBC at day 0</th>
<th>% biotinylated RBC at last detection</th>
<th>% of day 0 at last detection</th>
<th>Day of last detection</th>
<th>Linear Half-life (days)</th>
<th>Exponential Half-life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ</td>
<td>0.61</td>
<td>0.095</td>
<td>15.6</td>
<td>42</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>JC</td>
<td>0.63</td>
<td>0.01</td>
<td>1.6</td>
<td>14</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>KC</td>
<td>0.68</td>
<td>0.075</td>
<td>11.0</td>
<td>28</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>PS</td>
<td>0.42</td>
<td>0.08</td>
<td>17.3</td>
<td>49</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>OS</td>
<td>0.46</td>
<td>0.01</td>
<td>21.6</td>
<td>49</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>TK</td>
<td>0.53</td>
<td>0.09</td>
<td>12.7</td>
<td>35</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>VL</td>
<td>0.70</td>
<td>0.09</td>
<td>9.6</td>
<td>42</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>

5.4.4. Comparison of all patients and controls evaluated

Figure 5.4 displays the red cell survival curves determined for all patients and controls, so that comparisons can be made. The controls are shown in red, the β-thalassemia patients in blue, and the Hb E/β-thalassemia patients in green.
5.5 Comments on red cell survival studies

The use of *in vitro* biotin labeling to determine red cell survival in patients with thalassemia has not previously been reported. Studies with $^{51}$Cr labeling have shown survival times of autologous cells reduced by 30 - 70% (McCurdy 1969, Shahid 1971, Vigi 1969). The β-thalassemia patients in our study had red cell half-lives of 9 to 34 days, while red cell half-life in the Hb E/β-thalassemia patients ranged from 9 to 28 days. One control had a red cell half-life of 42 days, which was shown in two separate studies, while the second had a red cell half-life of 39 days.

Flow cytometric methods are only able to determine values of biotin labeling greater than 0.01%. Therefore, survival is considered complete at this point. However, since the amount of label found on day 0 varied considerably between patients (0.30 - 0.68%), this represented a different proportion of day 0 (which is assumed to be 100%) in each patient. The variation seen in day 0 labeling can be attributed to a number of causes,
including: variation in amount of red cells obtained for labeling, variation in efficiency of labeling, and variation in amount of blood volume of each patient (a larger blood volume would theoretically dilute the labeled cells which are re-infused, leading to a smaller proportion of labeled cells). Since the survival curves are based on percent of day 0 level, those that had higher levels of biotin of day 0 will have detection for longer, even though actual red cell survival time may be the same. Therefore, the red cell half-life provides a more accurate survival basis for comparison between patients.

The patients with β-thalassemia, who were studied first, began their studies when total hemoglobin concentration fell below 7.0 g/dL, without insisting on a maximum level of Hb A. As shown by the decrease seen in Hb A levels during the study, the three transfusion-dependent patients may have had significant amounts of transfused blood in their circulation, this may have artificially increased the observed survival times. The patients with Hb E/β-thalassemia were all weaned to a Hb A below 30%. The survival times in these patients were less likely influenced by transfused blood. The higher red cell half-lives seen in the patients with β-thalassemia, as compared to the patients with Hb E/β-thalassemia, may be an artifact of this.

None of the patients studied displayed negative effects due to the study, other than complaints of irritation at the site of the intravenous line, which cleared once the lines were removed. Venous access created a significant problem in only one patient (β-Thalassemia patient) in who venous access was lost after the 10 minute draw on day 0. Subsequent time points were missed and the patient was sent to the phlebotomy team for a final draw at 60 minutes. In this patient, day 0 value was a mean of these two values.

None of the patients included in this study have completed treatment with SPB and HU, therefore, post-treatment comparisons are not yet available.

Red cell biotin labeling has provided a viable method to determine red cell survival, without exposing patients to radioactive labels. Repeating these survival studies post-treatment will provide evidence of improved red cell survival following the augmentation of Hb F in patients with β-thalassemia and Hb E/β-thalassemia.
Chapter 6

General Discussion and Conclusions

6.1. General Discussion
With the introduction of regular red cell transfusions, \( \beta \)-thalassemia ceased to be a disease that almost invariably caused death within the first decade of life. However, the use of transfusions introduced a secondary disease, iron overload, which became the major cause of death (Bunn 1986). Treating \( \beta \)-thalassemia without relying on regular transfusions would lessen the iron overload experienced by patients. BMT offers curative treatment, but is restricted to those with an HLA-identical sibling; while, gene therapy holds promise but is not currently a viable option. This thesis examined two additional non-transfusion based treatment options: splenectomy and pharmacological augmentation of Hb F. The data presented suggest that these therapies may be effective treatments in some patients. Further study must be conducted to determine which baseline parameters, if any, predict response to these therapies.

6.1.1. Splenectomy in \( \beta \)-thalassemia intermedia and Hb E/\( \beta \)-thalassemia
Splenectomy provided an increase in total hemoglobin concentration in some transfusion-independent patients. However, it did not offer a consistent improvement in the extent of anemia, either when comparing individual patients pre- and post-splenectomy or when comparing the splenectomised patients to the non-splenectomised patients as a whole. This is consistent with what has been reported in the literature (Blendis 1974, Engelhard 1975, Fiorelli 1987, Piomelli 1995, Pippard 1982).

Patients with Hb E/\( \beta \)-thalassemia appeared to show a better hematological response to splenectomy, than did patients with \( \beta \)-thalassemia intermedia. The splenectomised patients in both centers studied (one in North America and one in Sri Lanka) did not appear to have significant adverse effects post-splenectomy.

6.1.2. Augmentation of Fetal Hemoglobin
The response to pharmacological augmentation of Hb F was also varied. Of the twelve patients who were evaluable, six had an increase in total hemoglobin concentration of at least 1.5 g/dL when treated with SPB and HU, while an equal number did not show a

This was the first formal analysis on the use of SPB and HU in combination. Four patients, who had not shown an increase in hemoglobin of at least 1.5 g/dL when treated with SPB alone, showed this response when HU was added to the treatment regimen.

This was also the first study to examine the use of SPB in infants with severe β-thalassemia. None of the five infants that were treated with SPB responded with a significant increase in total hemoglobin concentration and all were subsequently begun on regular red cell transfusions.

6.1.3. Predicting Response to Non-Transfusion Approaches
Since variations were seen in response to both splenectomy and treatment with SPB and/or HU, baseline(s) parameter that predicts response would be of clinical benefit, aiding clinicians in deciding which patients should be offered these therapies. However, no such parameters were elucidated through this thesis. Larger studies, which involved larger patient populations, may be helpful in determining if any such parameters exist.

6.2. Splenectomy in β-Thalassemia
6.2.1. Analysis Pre- and Post-Splenectomy
The major limitation of this study was the small sample size. Assuming that the minimum clinically significant increase in hemoglobin concentration between baseline and post-splenectomy is 1.5 g/dL, 21 patients are required for adequate analysis. Using a paired-sample t-test with a two-sided confidence level of 0.01 and an β error specification of 0.01, the following calculation is obtained:

\[
n = \frac{(Z_\alpha + Z_\beta)^2 \sigma^2}{\delta^2} = \frac{(2.58 + 2.33)^2 (1.4)^2}{1.5^2} = 21
\]

Where \( n \) indicates the sample size in the study group, \( Z_\alpha \) and \( Z_\beta \) respectively denote the upper \( \alpha \) and lower \( \beta \) percent points of the normal distribution, \( \sigma \) is the standard deviation expected for the mean difference in hemoglobin concentration pre- and post-
splenectomy, and \( \delta \) denotes the minimum clinically important difference in total hemoglobin concentration. The standard deviation used in this equation was obtained from the pre- and post-splenectomy analysis studies performed in this thesis.

In addition, as a retrospective study, all information had to be determined through a chart review. Therefore, the number of visits over which baseline and follow up parameters were gathered was different for each patient and it was not possible to independently verify the accuracy of the data.

A special problem in studying Hb E/β-thalassemia patients in Sri Lanka was the lack of evaluable hemoglobin concentrations analyzed by coulter. Prior to 1997, very few coulter hemoglobin concentrations were obtained, and even at the time of this study, the majority of hemoglobin concentrations are not obtained by coulter and are thus not reliable. Due to lack of coulter hemoglobins, none of the patients splenectomised more than two or three years before this analysis could be included, and many patients had been splenectomised within 12 months of this study.

Hematological improvement post-splenectomy may, in some patients, be temporary. As post-splenectomy analysis was limited to twelve months in the β-thalassemia intermedia patients, and from ten to 32 months in the Hb E/β-thalassemia patients, this may have been missed by the scope of the study.

Improvements in red cell survival have been reported following splenectomy. Also reported, post-splenectomy, are increases in hepatic iron concentration and PAP. These parameters were not analyzed in this study, as they were not available pre- and post-splenectomy in our patients.

6.2.2. Future Directions of Analysis Pre- and Post-Splenectomy
As stated above, a sample size of 21 patients is required for enough power to determine if splenectomy is able to increase hemoglobin concentration by at least 1.5 g/dL.

A prospective study of patients for 1 year pre-splenectomy and 5 fives post-splenectomy will aid in the analysis of the problems proposed in the previous section, dealing with the limitations of the current pre- and post-splenectomy analysis.
All patients would be seen in the clinic monthly pre-splenectomy, ensuring that all patients have a hematological baseline determine over an equal period of time. Patients will be monitored every 3 months post-splenectomy. Because decreased basal hemoglobin concentration has been attributed to decreased folate levels, all patients will be kept folate replete during the baseline and follow-up period.

To test the hypothesis that some patients may show only a temporary increase in hemoglobin concentration, patients will be followed for a minimum of 5 years post-splenectomy. This extended follow-up will also provide the opportunity to observe possible long-term complications.

At each clinic visit, patient height will be assessed. Growth velocity in the year prior to splenectomy will be compared to growth velocity in each year following splenectomy. Linear growth will also be plotted from each visit, to determine if it deviates from growth along the pre-splenectomy percentile.

All patients will undergo red cell survival studies, pre- and post-splenectomy to see if splenectomy increases red cell survival in these patients. In addition, quantitative hepatic iron concentration will be determined pre- and post-splenectomy. Pre-splenectomy analyses will be performed within 6 months of splenectomy; post-splenectomy analyses will be conducted 10 to 14 months post-splenectomy.

To determine whether pulmonary hypertension can be a complication of splenectomy, ECHO cardiograms will be performed pre- and post-splenectomy in all patients to determine PAP. To check if time since splenectomy increases the risk of pulmonary hypertension, the follow-up studies will be conducted yearly, during the five years of analysis.

This proposed study would have enough power to determine the effectiveness of splenectomy at increasing basal hemoglobin concentration. It will also allow for the comparison of linear growth red cell survival, hepatic iron concentration, and pulmonary hypertension, pre- and post-splenectomy.
6.2.3. Comparison of Splenectomised and Non-Splenectomised Patients

The study examined only the most recent 12 months available for each patient. It is possible that adverse effects (such as infections) were seen in the splenectomised patients during the years post-splenectomy that were not examined, and these were missed by the scope of the study.

Pulmonary hypertension has been reported as a complication of splenectomy, particularly in patients with persistent anemia. Even those of our splenectomised patients who showed a response to splenectomy had hemoglobin concentrations below normal, and it would thus be expected that at least some patients would exhibit pulmonary hypertension. However, not all patients had undergone right heart studies, so this comparison was weakened. Patients who had undergone right heart studies had them at varying times since splenectomy. The development of pulmonary hypertension may be seen only after time has elapsed since surgery; some patients may have been analyzed before this time had passed.

As stated above, in the pre- and post-splenectomy analysis study, improvements in red cell survival have been reported in splenectomised patients. Also reported in splenectomised patients are increased hepatic iron concentrations. These parameters were not available for examination in the patient populations studied, so the hypotheses could not be tested.

6.2.4. Future Directions of Comparison of Splenectomised and Non-Splenectomised Patients

In a comparison of splenectomised and non-splenectomised patients, I will conduct a five-year prospective analysis. All splenectomised patients will be matched with a non-splenectomised, age, sex, and genotype (i.e., $\beta^+$, $\beta^0$, Hb E/\(\beta\)) control.

These patients will be compared in terms of basal hemoglobin concentration, height velocity percentile, hepatic iron concentrations, and rate of infections. All patients will be monitored to ensure that they are folate replete, if not, they will be given supplemental folate.
Patients will be seen in clinic every 6 months for physical examination and laboratory tests. Hepatic iron concentrations will determined twice; once in the first year and again in the final year of analysis. Red cell survival studies will also be conducted once in the first year and again in the final year of analysis.

Right heart studies, through ECHO cardiograms, will be conducted yearly. This will allow for the comparison of the PAP between the splenectomised and non-splenectomised patients. The yearly testing will allow us to determine if time from splenectomy effects the development of pulmonary hypertension.

Linear height percentile and height velocity percentile will be compared between the two patients, yearly. Height percentile will be compared relative to mid-parental height.

This study will be conducted both in Toronto and in Sri Lanka. This would indicate if environmental factors (i.e., malarial infections) have an effect on the response to splenectomy in these patients.

6.3. Augmentation of Fetal Hemoglobin
6.3.1. Treatment with SPB and/or HU
Due to the high variability of response seen in patients treated with SPB and HU, the power of this study is limited and a larger patient population is required to make secure conclusions.

Assuming that the minimum clinically significant increase in total hemoglobin concentration between baseline and after treatment is 1.5 g/dL, a sample size calculation can be performed. Using a paired-sample t-test with a two-sided confidence level of 0.01 and an β error specification of 0.01, the following calculation is obtained:

\[
n = \frac{(Z_\alpha + Z_\beta)^2 \sigma^2}{\delta^2} = \frac{(2.58 + 2.33)^2 (1.3)^2}{1.5^2} = 19
\]

Where \( n \) indicates the sample size in the study group, \( Z_\alpha \) and \( Z_\beta \) respectively denote the upper \( \alpha \) and lower \( \beta \) percent points of the normal distribution, \( \sigma \) is the standard deviation expected for the mean difference in Hb F at baseline and after therapy, and \( \delta \) denotes the minimum clinically important difference in total hemoglobin concentration.
The protocol did not allow a plateau in the response to SPB to be reached before patients were begun on HU; they were begun on HU after three months of therapy with SPB regardless of the response seen to SPB. It is possible that patients who were beginning to show an increase in Hb F or total hemoglobin would have shown a significant rise in these parameters on SPB alone had they been allowed to continue without the addition of HU.

It was very difficult for transfusion-dependent patients to follow the protocol. The necessity of weaning from transfusions was difficult and some patients (4 of 11) withdrew from the study before beginning treatment. The overall dropout rate for transfusion-dependent patients was 81.8%, while the dropout rate for transfusion-independent patients was 30% (P=0.03).

The only method for determining patient compliance with therapy, in the treatment of infants with SPB, was parental history. SPB has a very bitter taste and it is difficult to get patients, particularly infants, to take the prescribed amount. Although parental history indicated a good level of compliance, it is likely that the patients were not receiving their proper dose. This may have adversely affected the responses seen.

6.3.2. Future Directions in Studying Augmentation of Hb F with SPB and/or HU
The study should be expanded to include patients from other centers in North America, providing a larger sample size, so that more secure conclusions can be reached. As noted above, a sample size of 19 patients is required for sufficient power to observe an increase in total hemoglobin concentration of 1.5 g/dL.

I propose treating all patients with SPB for 12 weeks. Any patient who has shown an increase in total hemoglobin concentration of 1.0 g/dL or greater would continue to receive SPB alone, until a plateau in total hemoglobin concentration is observed. At this point, HU will be added to the treatment regimen. The combination treatment would also be for a minimum of 12 weeks. Again, any patient who showed an increase in total hemoglobin concentration of at least 1.0 g/dL over that determined at baseline would continue on combination treatment until a plateau is observed. Once the plateau is reached, SPB would be removed and the patient would receive HU alone.
The study will be opened only to transfusion-independent patients, thereby removing the need to wean patients off of transfusions before initiating treatment.

It is possible that, in combination treatment, the order in which the drugs are introduced will be important. To test this hypothesis, I propose that the patients are randomized into one of two arms: in the first arm, patients receive SPB alone, followed by SPB and HU, and finally HU alone; in the second arm, patients receive HU alone, followed by HU and SPB, and finally SPB alone.

The analysis of α/non α globin chain synthesis ratios will be performed on the globin samples prepared from our patients. This will allow us to observe if the augmentation of Hb F was caused by upregulation of the γ-globin gene, as evidenced by a decrease in the α/non α globin chain synthesis ratios. This analysis will be performed as follows. Seven cc of whole blood are collected in heparin. The blood is centrifuged at 1500 rpm for 10 minutes at 4°C. The buffy coat is removed, and reticulocytes isolated by washing the sample twice in reticulocyte buffered saline (RBS); the red cells are washed in 12 mL RBS and spun for 10 minutes at 1500 rpm twice. The cells are resuspended in 12 mL RBS and spun at 3000 rpm for 30 minutes at 4°C. The supernatant is discarded and 0.5-mL of the reticulocyte-enriched cells are removed from the top of the spun cells. These cells are incubated for one hour at 37°C in reticulocyte media with 2 drops of 1 mg/mL ferrous ammonium sulphate and 50 μCi ³H-leucine. Incubation is stopped by the addition of 20mL ice-cold RBS. The cells are centrifuged at 1500 rpm for 10 minutes at −20°C and supernatant discarded in order to remove the free radioactivity. The cells are lysed by the addition of 2 volumes of distilled water. Twenty volumes of 2% concentrated HCl in acetone at −20°C are added to the lysed cells. Acetone removes the globin from the hemoglobin molecule by destroying the hydrogen bonds between heme and globin and the acid then precipitates the globin. To ensure complete precipitation, the mixture is allowed to stand at −20°C for 20 minutes. The suspension is then centrifuged at 3000 rpm for 3 minutes at −20°C. The acid-acetone supernatant is discarded. The globin is washed with 45 mL of acetone and spun at 3000 rpm for 3 minutes at −20°C, three times. This is done to remove all traces of heme and acid. A final wash is done with 45 mL diethyl ether. The globin is allowed to dry completely at room temperature, before being stored at −20°C.
Although not yet performed, the globin chains can be separated, to determine globin chain ratios. CM-23 cellulose column chromatography is used to separate the globin chains. The chromatography is performed using a urea-phosphate buffer gradient. A pH between 6 - 7 is optimal for globin chain separation, but at this pH, urea is needed as a solvent to dissolve the globin powder. The ability of the globin chains to bind the CM-23 cellulose is dependent on the positive charge of the chain. At pH 6-7, the relative affinities of the four chains for CM-23 cellulose are \( \alpha > \delta > \beta > \gamma \). Therefore, the \( \gamma \)-chain elutes first, followed by \( \beta \), \( \delta \), and finally \( \alpha \). Sixty to 70, 5 mL fractions are collected at 10-minute intervals for 14 - 15 hours. Aliquots from each fraction are mixed with scintillation liquid and incorporated radioactivity is measured in a liquid scintillation counter. A plot of new cpm for each tube is plotted against fraction number and the \( \gamma \), \( \beta \), and \( \alpha \) peaks are determined. The background level (average count in area before peaks) is subtracted from the area under the curve for each peak. The resulting values are then compared to each other to determine the globin chain ratios.

This proposed study would have enough power to determine if treatment with SPB and/or HU can provide clinical benefit to patients with \( \beta \)-thalassemia. It will also provide insight into the affect of age and order of drug administration on the response seen. The use of \( \alpha \)/non \( \alpha \) globin chain synthesis ratios will provide evidence that the clinical effect is a result of \( \gamma \)-globin augmentation.

### 6.4. Red Cell Survival

Of the eleven patients (four with \( \beta \)-thalassemia and seven with Hb E/\( \beta \)-thalassemia) that were included in the red cell survival study, ten of them were transfusion-dependent. Although these patients were weaned from transfusions, it was not feasible to wean them to their true baseline hemoglobin concentration. Therefore, the red cell survival studies were performed with varying amounts of transfused blood still in these patients, so not all of the biotin labeled cells were truly autologous cells.

A larger amount of labeled red blood cells reinfused increases the absolute amount of biotin labeled red blood cells on day 0, determined through day 0 draws (although the values for all patients were converted to 100% for day 0). Analysis by FACS is only able to detect biotin-cells at concentrations greater than 0.01%. Depending on the amount of
cells labeled on day 0, this represents a different proportion of the initial label. Therefore, in patient KC, in whom 0.7% of the cells were labeled on day 0, this represented 1.5%, while it represented 3.3% in patient TC, in whom 0.3% of the cells were labeled on day 0. Therefore, measurable red cell survival represented a different proportion of true survival for each patient.

As shown by Mock (1999), in vitro labeling of red blood cells leads to slight damage of the red blood cells, reducing the calculated red cell survival time (Mock 1999); therefore, the survival times recorded for patients in our study may be erroneously low.

6.4.1. Future Directions of Red Cell Survival Study

Biotin-labeled red cell survival studies conducted on more patients will give a clearer picture of the survival time seen in patients with thalassemia. Attempts to correlate red cell survival with other clinical parameters of disease (basal hemoglobin concentration, transfusion requirements, hepatosplenomegaly, etc) will provide insight into disease pathophysiology.

All patients who underwent red cell survival studies were enrolled in protocols to examine the pharmacological augmentation of Hb F. A repeat of the survival studies after treatment will provide an opportunity to correlate the increases seen in total hemoglobin concentration and Hb F concentrations with improvements in red cell pathophysiology, as demonstrated by red cell survival.


Chapter 8

Appendix

8.1. Study Forms

RESEARCH INFORMATION FORM

Title of Research Project:

Augmentation of Fetal Hemoglobin in β-thalassemia

Investigator
Dr. Nancy F. Olivieri
Professor of Medicine and Pediatrics
Division of Haematology/Oncology
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Purpose of the Research:

This study is being conducted to test the effectiveness of several drugs to increase a type of hemoglobin in the red cells in your blood. Thalassemia, or Cooley's anemia, is a blood disease in which regular blood transfusions are usually required. In all people within the first year of life, the 'switching' from 'fetal' hemoglobin (Hb F) to 'adult' hemoglobin (Hb A) occurs. Normal amounts of Hb A are not produced in people with thalassemia, but if it was possible to reactivate Hb F in thalassemia, it might be possible to reduce, or eliminate, blood transfusions. Treatment of some patients with two drugs, hydroxyurea (HU) and sodium phenylbutyrate (SPB), have shown that, alone and in combination, these drugs have successfully turned on Hb F so that in some, but not all, treated patients, blood levels have increased significantly. Treatment of other patients with a hormone called erythropoietin (EPO) has also successfully turned on Hb F so that in some, but not all, treated patients, blood levels have increased significantly. Doctors are not sure which combinations of these drugs is the best way to increase Hb F.
Description of the Research

A number of patients will be enrolled in this study. If you agree to participate, your involvement will last approximately 24 months.

A. Stopping Transfusions
If you agree to take part in this study, and are currently receiving regular blood transfusions, you will gradually receive fewer blood transfusions until they are stopped completely. In the absence of blood transfusions your bone marrow may be more likely to respond to these medications. Because you will not be receiving the same frequency of transfusions, your blood counts may fall slightly and you may feel tired. If at any point during the study your hemoglobin falls below 7 grams/deciliter, or if you feel tired or otherwise unwell, you will be offered a blood transfusion.

B. Medication
There are 5 treatment phases whereby each drug is taken alone or in combination with another medication. The first treatment phase uses sodium phenylbutyrate (SPB) for 3 months. The second treatment phase adds hydroxyurea (HU) to sodium phenylbutyrate (SPB) for 3 months. In the third treatment phase, sodium phenylbutyrate is withdrawn and hydroxyurea is used alone for 6 months. In the fourth treatment phase erythropoietin (EPO) is added to hydroxyurea for 3 months. In the fifth treatment phase

C. Measuring Red Cell Survival
Study of the red cell survival is not required for you to agree to participate in the treatment study. Therefore, there is a separate form for the red cell survival study, which is attached.

The study of the red cell survival is done before starting any medication. If you agree to take part, you will come to the hospital and have an intra-venous catheter placed in your arm. During this visit, which will last for approximately 4 hours, there will be several blood tests drawn:

1) First, about three teaspoons of blood will be drawn. This blood will be taken to the laboratory, using a method to keep your cells sterile (free of infection). A "label"
called biotin will be added to the red blood cells in this sample of blood. This label allows doctors to estimate the life span of red blood cells.

2) After the "label" is added to the sample of your blood, the same samples will be given back to you through the same intra-venous catheter from which it was drawn.

3) Next, the doctor or nurse will draw 8 more blood samples, each about a teaspoon, from the catheter over the next 4 hours. After the last blood sample is drawn, the catheter will be taken out, and you will be able to go home. This will take a total of about 4 hours of clinic time.

4) After the first few days, as long as you still have some labeled cells in your blood, you will have to come back only once a week for six to eight weeks to have one teaspoon of blood drawn from each time.

This procedure will be repeated at the end of the study.

D. Physical Exam
At the start of the study you will be seen by a physician for a physical exam. You will have about 2 tablespoons (30 ml) of blood taken by needle from a vein. If a vitamin called folic acid is low in your blood, you will be asked to take folic acid daily throughout the study. You will be given sodium phenylbutyrate by mouth every day, about 10-20 pills per day, spread over the day.

After three months, hydroxyurea will be added to treatment with sodium phenylbutyrate. The dose of the hydroxyurea will be increased every 8 - 12 weeks until the highest dose you can safely take is reached. You will be treated for at least 3 months at this dose, which is called your "maximum tolerated" dose. While taking hydroxyurea, 2 teaspoons of blood will be drawn from a vein or by fingerprick every two weeks to check for changes in the blood counts.

Pregnancy:
If you are female, you must not be breast-feeding, pregnant, or planning to become pregnant during the study. If you are sexually active, you must agree to use an effective
form of birth control. Pregnancy tests will be done monthly on sexually active females. If you are male and sexually active, you must also agree to use birth control.

Clinic Visits:
Every four weeks, you will have an assessment with the doctor and research coordinator. There will be a physical examination, blood work and a review of how well you are tolerating the medication. Hydroxyurea will be stored in a special pill container that has a computer chip in the cap. This chip records how often you are taking the medication.

Potential Harms (Injury, Discomforts or Inconvenience)

1. The use of a catheter may cause a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness that usually disappears in a few days.

2. The "labeling" of the blood will be done in a completely sterile (non-infectious) environment. There is a small chance of infection after the blood is re-injected.

3. You will also have the inconvenience of having to come to the hospital frequently at the beginning of the study.

4. Hydroxyurea has been shown to be safe with close monitoring of blood counts. Sometimes hydroxyurea can cause a fall in white cell counts if too much of the drug is given. Blood counts usually return to normal after a week or two when hydroxyurea therapy is stopped. If blood counts drop during therapy, hydroxyurea will be immediately stopped, and once the counts return to normal, hydroxyurea will be started at a lower dose.

5. Worries that long-term hydroxyurea treatment may be associated with an increased risk of cancer have not been shown to be true in large numbers of patients with several types of diseases, some of whom have been treated with hydroxyurea for more than ten years.
6. Other side effects of hydroxyurea that have been reported include upset stomach (which may be improved by taking the medication just before bedtime), hair loss, skin rash and drowsiness. None of these are proven, and all were just as common in patients not receiving hydroxyurea in a large study. All reported side effects disappeared after stopping the medication.

7. Hydroxyurea may be associated with other risks that are unknown at present, and which doctors cannot predict.

8. If any significant new findings develop during the course of the research that may affect your willingness to continue in the study, we will notify you as quickly as possible.

9. Sodium phenylbutyrate has no known risks but may be associated with swelling of the ankles, rash or a strange body odor.

10. Recombinant erythropoietin has no known risks but because it is given under the skin with needles, may be irritating to some patients.

11. The effect of all these drugs on an unborn baby is unknown, so that your participation in the study will be immediately interrupted if you become pregnant during this study, or make someone pregnant. The most complete advice available will be given to you.

12. If you feel very weak and tired without regular transfusions, you will be treated with red cell transfusions in small amounts. You will not be withdrawn from the study if you require transfusions, but may continue if you wish.

Potential Benefits

Early studies of sodium phenylbutyrate, hydroxyurea, combination therapy with hydroxyurea and sodium phenylbutyrate, and erythropoietin have observed increased in the levels of Hb F, so that some patients no longer needed blood transfusions or iron-chelating therapy with deferoxamine and their Hb F increases. This means that if these
drugs work in you, you may become free of transfusions during this therapy. On the other hand, you may not respond from the use of these drugs.

The study of red cell survival will not benefit you directly. Nevertheless, it will give doctors information about red blood cells in thalassemia patients and the effect of hydroxyurea and sodium phenylbutyrate treatment. This may help the doctors understand how to use these drugs in a better way.

Alternatives

Whether or not you choose to participate in this study, you will continue to receive up-to-date health care at The Hospital for Sick Children.

The alternatives to entering this study include continued red cell transfusion and iron-chelating therapy. As noted, this study is being conducted to explore alternative methods of treatment that are, at this time, experimental in nature.

Confidentiality

Confidentiality will be respected and no information that discloses the identity of the subject will be published without consent unless required by law. You will not be identified personally in any report from this study. Your personal medical records will be kept private. Information may be given to the U.S. National Institutes of Health or the U.S. Food and Drug Administration for the purpose of monitoring the study, but your name will not be used in such files. For your information, the research consent form will be inserted in the patient health record.

Reimbursement

There is no payment to participants in this study. The medication (SPB, HU and EPO) will be provided at not charge to you.
Compensation

In the event that this research activity results in an injury, treatment will be available, including first aid, emergency treatment, and follow-up care as needed. Payment for any such treatment will be provided by OHIP.

Participation

Participation in research is voluntary. If you do not choose to participate, you and your family will continue to have access to quality care at The Toronto Hospital, General Division. If you agree to participate in this study, you may withdraw from the study at any time. Agreement to any of the drug treatments does not require that you agree to take any of the later drug treatments. Again, you and your family will continue to have access to quality care at The Toronto Hospital, General Division.

If you have any questions, either before deciding whether to participate or during the course of this study, please direct your questions to Dr. Nancy Olivieri (813-6823). Additionally, if you wish to speak to a physician who is not involved with this research project and is available for reference, you may contact Dr. Ron Heselgrave Chair of the Research Ethics Board of The University Health Network, at 416-340-4557.