HEPATIC IRON QUANTITATION AND LIVER BIOPSY IN SICKLE CELL DISEASE AND THALASSEMIA MAJOR:

IMPACT ON MONITORING AND PREVENTING THE PROGRESSION OF IRON OVERLOAD DUE TO REGULAR TRANSFUSION THERAPY

by

Sergio Muraca

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Institute of Medical Science
University of Toronto

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Hepatic Iron Quantitation and Liver Biopsy in Sickle Cell Disease (SCD) and Thalassemia Major (TM):
Impact on Monitoring and Preventing the Progression of Iron Overload due to Regular Transfusion Therapy

ABSTRACT

Iron accumulates and becomes toxic in transfused patients with SCD or TM. Quantitative hepatic iron concentration (HIC) using liver biopsy tissue optimizes accuracy of iron measures. The objectives of this study include determining the safety of liver biopsies, rates of iron accumulation, reliability of serum ferritin concentrations (SFC), incidence of liver damage, and efficiency of chelation therapy. From a series of 911 biopsies, 98% captured adequate tissue for analysis, and only 7 minor complications occurred (0.77%). In a second series of 18 SCD and 22 TM children, biopsies and SFCs following an initial period of transfusions revealed similar rates of iron accumulation and liver damage in both disorders. HIC exceeded thresholds of risk following 13 months of transfusions, or 110–130ccRBC/kg. A poor HIC/SFC correlation was observed. In a third series, records from 35 TM patients treated with deferoxamine or deferiprone were used to calculate chelation efficiency, revealed to be 15.1% and 2.6%, respectively.
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ABBREVIATIONS

CT = computed tomography
DFO = deferoxamine (desferrioxamine B)
Fe = iron
Hb = hemoglobin
HbA = normal adult hemoglobin
HbS = abnormal sickle hemoglobin
HCV +/- = hepatitis C virus positive or negative
HIC = hepatic iron concentration
L1 = deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one)
MRI = magnetic resonance imaging
RBC = red blood cell
SCD = sickle cell disease
SFC = serum ferritin concentration
SQUID = superconducting quantum interference device
TBIS = total body iron store
TM = thalassemia major
US = ultrasound
1.1 Iron Metabolism in Humans

1.1.1 Nature and Quantity of Iron Compounds

Iron is the 26th element of the periodic table, has an atomic weight of 55.85, and is the fourth most abundant element on and within the earth. Due to its bioavailability and biochemical properties, iron has become a participant in various living processes. The ability of an iron molecule to fluctuate between a reduced ferrous (Fe\(^{2+}\)) and oxidized ferric (Fe\(^{3+}\)) state, makes it ideal for participating in oxygen- and electron-transport, and for catalyzing oxidation-reduction reactions. However, free elemental iron is cytotoxic, binding nonspecifically to many proteins altering their structure and function, and catalyzing oxidation reactions, such as peroxidation of unsaturated lipids in cellular membranes (Finch and Huebers, 1982). Therefore, iron must be bound to a functional, storage, or carrier protein, in order to avoid its deleterious effects on cells and tissues.

Normal total body iron concentration is approximately 40 mg/kg in adult females, and 50 mg/kg in adult males (Finch and Huebers, 1982; Israels and Israels, 1996). Thus, a normal 70 kg male human body contains about 3.5 grams of iron. A dynamic equilibrium maintains approximately 75% of total body iron (35-40 mgFe/kg) within functional compounds, and approximately 25% of total body iron (10-15 mgFe/kg) within intracellular storage depots (Bothwell et al., 1979). The minute but dynamic circulating plasma pool contains approximately 0.1% of the total body iron (0.05 mgFe/kg) normally involved with iron transport via transferrin protein molecules.

![Figure 1.1. Normal distribution of total body iron in man.](image-url)
1.1.1.1 Functional Iron

In the human body, iron is distributed and in a dynamic equilibrium between three principle compartments, functional iron compounds, iron in transit bound to transferrin, and iron storage compounds. Altogether, the normal human body incorporates approximately 75% of total body iron, equivalent to 35 to 40 mg Fe/kg, within functional compounds (Bothwell et al., 1979; Finch and Huebers, 1982). Functional iron compounds include (1) the heme complexes of hemoglobin, myoglobin, and various heme-containing enzymes, and (2) non-heme iron-containing proteins and enzymes.

In the normal human body, almost 90% of functional iron, or approximately two-thirds of total body iron, (equivalent to between 27 and 33 mg Fe/kg) is incorporated within the heme complexes (Figure 1.2) of the oxygen-transport hemoglobin molecules within red cells (Israels and Israels, 1996). Myoglobin molecules, heme-iron-containing oxygen-transport proteins (similar to hemoglobin) found in muscle fibers, contain about 7-8% of total body iron, equivalent to 3 to 4 mg Fe/kg (Barry, 1973). The remainder of functional iron, approximately 4% of the total body iron (equivalent to 2 mg Fe/kg), is distributed among various heme and non-heme containing enzymes. including the cytochromes, the iron-sulfur proteins of respiration, the metallo-flavoproteins and other enzymes in which iron is a cofactor.

![Structure of a pyrrole ring](image1)

![Abbreviated version of pyrrole](image2)

![Heme molecule](image3)

Figure 1.2a & b. Heme is a member of a family of compounds called porphyrins. Four weakly aromatic pyrrole rings joined by methene bridges comprise the structure of protoporphyrin IX. Iron (II), or ferrous iron, added to protoporphyrin IX produces heme (Baggott and Dennis, 1995).
1.1.1.2 Iron in Transit

Transferrin is the principle iron transport protein, normally carrying approximately 4mg of iron at any given moment, but cycling and redistributing over 30mg of iron daily (Huebers and Finch, 1984). Transferrin receives iron (1) from dietary sources (~1mg/day), (2) recycled from old and defective red cells by reticulo-endothelial macrophages (~25mg/day), and (3) released from tissue stores (~5mg/day). Transferrin delivers over 80% of this iron to the bone marrow to be reutilized during erythropoiesis, and redistributes the excess iron into parenchymal iron stores, mainly within hepatocytes. Immature erythroid cells and hepatocytes have a large number of transferrin receptors on their surface to accept and internalize transferrin-bound iron.

1.1.1.3 Reserve / Storage Iron

In the normal human body, iron that is not serving a functional purpose, and not in transport bound by transferrin, is stored in various tissues within intracellular deposits of either soluble ferritin or insoluble stainable hemosiderin molecules. Ferritin and hemosiderin are storage molecules specially designed for holding iron in a non-reactive form in a readily available reserve. The principal iron storage pools are located in the liver, spleen, and bone-marrow (Bothwell et al., 1979; Barry, 1973).

In the normal human body, an average of approximately 25% of total body iron (equivalent to 10 to 12 mgFe/kg, ranging between 0 – 20 mgFe/kg), remains stored within ferritin (~9 mgFe/kg) and hemosiderin molecules (~4 mgFe/kg) (Israels and Israels, 1996; Bothwell et al., 1979). The storage compartment is unique among the other body iron compartments, due to its capability of significant expansion. However, in healthy males the amount of iron contained in reserve remains constant throughout life, reflecting the normal equilibrium between the controlled absorption of dietary iron and the relatively uncontrollable physiological iron loss. In females, menstruation and pregnancy cause variability or fluctuations in the amount of iron contained in reserve. Perhaps attributable to processes of evolution, the normal amount of iron stored in the body varies among different populations.
1.1.2 Iron Availability and Absorption

Iron absorption is the primary regulator of iron metabolism and storage. The average diet contains 15mg of iron per day, of which approximately 3 mg is internalized by the cells of the duodenum and proximal jejunum, and about 1-2 mg is transferred into the plasma (Harford et al., 1994). As total body iron levels decline, through normal physiological iron loss or hemorrhagic blood loss, iron absorption is increased to maintain or re-establish normal iron levels. Likewise, as body iron levels increase (ie. through repeated blood transfusions), the body shuts down gastrointestinal iron absorption in an effort to suppress iron accumulation. Normally, physiological iron losses are small, averaging approximately 1.5 to 2mgFe/day in menstruating females, and 1 mgFe/day in males and non-menstruating females (Israelis and Israelis, 1996). In normal response, the body absorbs 1 to 2 mg/day of dietary iron. During pregnancy the female body increases iron absorption to 4 – 6 mgFe/day (Israelis and Israelis, 1996).

An appropriate diet provides approximately 15mg/day of iron, although less than 10% of this iron is sufficient to maintain iron balance, and actually gets absorbed. There are two major forms of dietary iron, which are absorbed by the digestive tract through different mechanisms. Heme iron, found primarily in red meats, is the most easily absorbed form, and approximately 20-30% of dietary heme-iron is absorbed from the gut. In comparison, about 5% of dietary non-heme iron, usually bound to some other organic constituent of the food, is absorbed. Cooking tends to break these interactions and increase iron availability. Furthermore, some iron-rich foods are poor sources of iron because other compounds render it non-absorbable.

1.1.2.1 Non-Heme Iron Absorption

The absorption of non-heme iron is influenced by the poor bioavailability of non-heme iron compounds in the diet. Furthermore, absorption of non-heme iron is influenced by inhibitory and enhancing substances (ie. ascorbic acid) present in food. Non-heme iron ions undergo two important changes of Oxidation State during digestion.
and absorption. The first change occurs in the stomach, where the low acidic pH causes non-heme iron to dissociate from any bound ligands and favors the reduction of ferric iron (III) to ferrous iron (II). Reducing agents, such as ascorbic acid, assist this process. Reduction is important because iron (II) dissociates from ligands more easily than iron (III). The second change occurs in the duodenum and proximal jejunum, the major sites of iron absorption, which are bicarbonate-rich, and alkaline. As this iron passes into the duodenum and jejunum, free ferrous iron (II) ions are oxidized to ferric iron (III), and are bound by special receptors on the brush border of intestinal mucosal cells (Figure 1.3). The iron is internalized and transferred to intracellular transport proteins. Iron is then either shuttled through the cell and released into the plasma (ferric iron (III) must be reduced to ferrous iron (II) within the mucosal cell in order to cross the plasma membrane), or alternatively iron may be deposited and stored as ferritin, which may eventually be eliminated through cellular exfoliation.

![Image](image.png)

**Figure 1.3.** Iron Absorption Events in the Stomach and Duodenum (Baggott and Dennis, 1995)

**1.1.2.2 Heme Iron Absorption**

Heme is not degraded in the lumen of the gut, and in the alkaline environment of the duodenum and jejunum heme is absorbed by intestinal mucosal cells with iron still
locked within the porphyrin ring. The absorption of heme by these mucosal cells is not influenced by other dietary factors or gastrointestinal secretions. Within intestinal mucosal cells, heme oxygenase drives the dissociation of ferrous iron (II) from heme. Intracellular transport proteins then shuttle the iron through the cell and either release the iron into the plasma, or store the iron within ferritin depots. Once again, the stored iron may eventually be eliminated through cellular exfoliation.

1.1.2.3 Regulation of Iron Absorption

Several factors or physiological situations may influence the absorption of iron. Ineffective and hyperactive erythropoiesis, either pathological (eg thalassemia) or as a result of bleeding or hemolysis, is associated with enhanced iron absorption, regardless of the presence of iron overload (Israels and Israels, 1996).

The amount of storage iron in the body will influence iron absorption. The body responds to an increase in the size of iron stores by suppressing iron absorption. Iron absorption is also suppressed in response to the inhibition of erythropoiesis caused by hypertransfusion (Israels and Israels, 1996).

1.1.2.4 Exogenous Iron Ligands

The dietary inhibitors of iron absorption that are listed in Table 1.1 form large insoluble polymeric complexes with iron. The dietary promoters of iron absorption form soluble monomeric complexes with iron. Alcohol (*) is not a ligand, but is a promoter of absorption by stimulating gastric secretions.

<table>
<thead>
<tr>
<th>Table 1.1. Exogenous Dietary Influences of Iron Absorption</th>
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<tr>
<td><strong>Dietary Inhibitors of Absorption</strong></td>
</tr>
<tr>
<td>Carbonates</td>
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<tr>
<td>Oxalates</td>
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<tr>
<td>Phosphates</td>
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<tr>
<td>Tannates</td>
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<tr>
<td>EDTA (food preservative)</td>
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<td>Clay</td>
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<td>Clay</td>
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1.1.3 Iron Loss

In normal human adult males and non-menstruating females, the rate of total body iron loss is 1 mgFe/day. In menstruating females, the average rate of iron loss increases to between 1.5 to 2 mgFe/day (Israels and Israels, 1996). There are three principle sources of iron loss. Exfoliation of all types of epithelial cells from their surfaces (ie. skin, GI tract, GU tract) is a major source of iron loss. Iron is contained within these cells in the form of intracellular enzymes and ferritin. Although the amount of iron contained within each epithelial cell is miniscule, due to the large number of epithelial cells that are shed each day, the combined effect is a very significant daily iron loss. Physiological hemorrhage and blood loss (ie. menstruation and trauma) is another major source of iron loss in humans. The third major source of iron loss in humans, which is relatively minor in comparison to epithelial exfoliation and hemorrhage, is through various bodily secretions, including sweat, bile, and urine, which each contain low concentrations of extracellular iron.

1.1.3.1 GI Tract

The human bowel has evolved with a very limited capacity to excrete iron, so the control of iron balance is much more dependent on absorptive mechanisms rather than excretion. The rate of gastrointestinal endogenous iron excretion has been quantified as approximately 0.6mgFe/day. There are three major sources of iron loss through the GI tract. Blood, normally present in human feces, contributes the largest portion of iron lost through the GI tract. Although much of the hemoglobin iron is reabsorbed, approximately 0.4mgFe/day escapes within fecal blood. Mucosal exfoliation contributes a daily loss of approximately 0.14 mg of iron, as iron from the plasma pool is absorbed by mucosal epithelial cells and is lost through cellular exfoliation (Green et al., 1968). Bile is the third major source of iron loss in the GI tract. The volume of bile that is secreted daily into the GI tract contains approximately 0.24 mg of iron. However, much of this biliary iron is re-absorbed by the GI tract (Bothwell et al., 1979).
1.1.3.2 Skin

Desquamation of the epidermis contributes 0.2 – 1.2 mg/day to iron loss. Endogenous iron enters the skin from the plasma pool. Iron is also excreted in sweat, with a daily loss between less than 0.1 mg/day to as much as 0.5 mg/day.

1.1.3.3 Urine

A minor source of iron loss is the urine, which normally contributes no more than 0.1 mgFe/day. Iron is present within voided erythrocytes, leukocytes, and epithelial cells of the GU tract. Furthermore, ferritin and other iron-containing compounds have been detected in the urine, which may have been from cells that have disintegrated during or after exfoliation.

1.1.3.4 Menstruation

Menstruation contributes a monthly iron loss of approximately 12 – 15 mg, which is equivalent to a rate of between 0.4 - 0.5 mgFe/day. Heavy menstrual blood loss is experienced in approximately 10% of women, resulting in an elevated rate of iron loss potentially greater than 1 mg/day. Undoubtedly, menstruation can be a major factor in the pathogenesis of iron deficiency.

1.1.3.5 Daily Rate of Iron Loss

In adult males and menopausal females, the total rate of iron loss is an average of 0.9-1.0 mg/day. Two-thirds of the total iron loss (~0.6mg/day) is contained within feces, while urinary excretion contributes less than 0.1 mg/day. The remainder of iron loss is through skin exfoliation and sweat, contributing an average of 0.2 – 0.3 mgFe/day. In pre-menopausal adult females, menstruation contributes an additional 0.6mgFe/day, raising the daily rate of total iron loss to 1.5 - 1.6 mg/day.
1.1.3.6 Influence of Iron Stores

In iron deficient states, iron loss can be minimized to approximately 0.5 mgFe/day (Dubach et al., 1955). In states of iron overload, iron excretion can be increased to 1.5mg/day, mainly through the suppression of excreted iron re-absorption mechanisms (Bothwell et al., 1979).

1.1.3.7 Iron Losses in Infants and Children

Normal infants and children lose 2 - 3 times more daily iron per kilogram body weight than adults do. In 1964, Garby et al quantified a rate of 0.03 mg/kg/day of iron loss in infants (Garby et al., 1964). In 1966, Elian et al reported an increased concentration of gastrointestinal iron loss in infants and children compared to adults, after observing relatively greater amounts of fecal blood contributing 0.25mgFe/day to a fecal iron loss (Elian et al., 1966). The fact that the surface area of the gut of the newborn is as much as 25% greater than that of the adult (Crelin, 1973).

1.1.4 Plasma Iron and Iron Transport

1.1.4.1 Normal Plasma Iron Distribution

Normally, more than 95% of plasma iron is bound to apotransferrin, the iron-transport protein. This pool of transferrin-bound plasma iron is referred to as "plasma iron" or "serum iron". Approximately 2% of the iron in the plasma is bound to hemoglobin molecules freely circulating in the plasma. The sources of this hemoglobin include intravascular hemolysis and the lysis of red blood cell precursors in the bone marrow. Haptoglobin is a special carrier protein circulating in the plasma that binds plasma hemoglobin and transports it to the liver for processing. If the concentration of plasma hemoglobin exceeds the carrying capacity of haptoglobin, the hemoglobin molecules may dissociate and another plasma protein, haemopexin, binds the heme molecule. Any additional iron present within the plasma, usually less than 1%, is either
associated with ferritin, or trace amounts of iron may be bound to lactoferrin, albumin, citrate, and ascorbate.

1.1.4.2 Transferrin

Once iron (II) crosses the mucosal cell membrane and enters the plasma, it is quickly reoxidized to iron (III) by the ferroxidase II enzyme. Iron (III) is then bound and transported by the serum protein, transferrin, also known as siderophilin or β1-metal combining globulin (Figure 1.4).

![Figure 1.4. Uptake of iron into the blood by transferrin (Baggott and Dennis, 1995)](image)

Transferrin is an 80 000 kD single-chain polypeptide glycoprotein largely synthesized in the liver. Transferrin has a bi-lobed structure containing two high-affinity and highly specific iron-binding sites, each capable of tightly binding one molecule of iron (III), using carbonate or bicarbonate as a synergistic anion (Gorinsky, 1982). Transferrin is the protein responsible for distributing iron throughout the body in proportion to need, which includes the iron requirements of the erythroid marrow for hemoglobin synthesis. Transferrin is also the transport protein that carries and deposits iron in the tissues adapted for iron storage, effectively minimizing the loss of iron from the body. Since transferrin functions as a true carrier molecule, it is not destroyed or altered during iron distribution, and has a half-life of 8 days. The normal concentration of serum transferrin is approximately 224 mg/dL, or between 24.0 to 48.0 μmol/L of
blood, providing a total iron binding capacity capable of binding approximately 300 μg/dL (60 μmol/L) of iron. About 4/9 of transferrin molecules are circulating in apoferic form, with no iron bound at either sites, another 4/9 have iron bound at one site (monoferric transferrin), and only about 1/9 have iron bound at both sites (diferric transferrin). Therefore, transferrin is normally only about 1/3 saturated with iron, carrying 100 μg/dL (20 μmol/L) of serum iron, and there is a substantial unsaturated plasma iron binding capacity (Israels and Israels, 1996). Therefore, an unexpected influx of iron can be handled easily.

1.1.4.3 Plasma Hemoglobin Iron

The normal plasma hemoglobin concentration is relatively minute at 4.5mg/dl of blood. This is equivalent to approximately 1.5 μgFe/dL. Normally, almost all this plasma hemoglobin (>99%) is bound to haptoglobin. Unbound plasma hemoglobin tetramers dissociate into dimers, which are filtered through the glomeruli of the kidney and excreted in the urine. Haemopexin binds to heme molecules, not hemoglobin, and removes them from the circulation as they are delivered to hepatocytes.

1.1.4.4 Plasma/Serum Ferritin

Two principle sources of plasma or serum ferritin have been proposed: (1) secretion from RE cells, and (2) leakage from damaged cells. The principal source of plasma ferritin appears to be the cells of the reticulo-endothelial system (Worwood, 1982). Non-viable red blood cells are phagocytosed and destroyed by monocytes and macrophages, and the RE cell acquires the iron released as the hemoglobin within the erythrocytes is degraded. This iron may be deposited within ferritin protein synthesized by the RE cell, a proportion of which may be released by the RE cell into the plasma as iron stores become saturated (Worwood, 1982). Damaged parenchymal cells containing massive deposits of storage iron are another source of plasma ferritin, as intracellular ferritin leaks through deteriorating cell membranes into the plasma (Worwood, 1982).
The iron content of serum ferritin is very low (measured at 23 and 67 \( \mu \text{gFe/mg of protein} \) in 2 iron overloaded patients), and significantly lower than the iron content of tissue ferritin (approximately 250 \( \mu \text{gFe/mg of protein} \) in iron overloaded patients) (Worwood, 1982).

1.1.5 Storage Iron

Iron is essential for life and must be conserved, however unbound ionic iron is toxic. Therefore, any iron that is a surplus to the immediate functional need, is transferred to iron stores. In humans, iron is stored in two major forms, incorporated within ferritin molecules as a diffuse, soluble, and mobile fraction, or it may aggregate into insoluble deposits of hemosiderin. Reticulo-endothelial cells and hepatocytes are the two major compartments of iron storage (Finch et al., 1984), however the mechanism of iron uptake into these compartments is quite different.

1.1.5.1 Ferritin

The major form of iron storage is within ferritin, a complex of 24 polypeptide subunits with a molecular weight of approximately 450 000 (apoferritin), arranged as a spherical shell with an outer diameter between 12 – 14 nm and inner diameter of 7.5 nm (Harrison et al., 1980). Ferritin is visualized only using electron microscopy. Six hydrophobic channels, approximately 1nm wide through the ferritin shell, allow the passage of iron into the protein core. Ferritin has a capacity of about 4500 iron (III) ions per molecule. However, fully iron-saturated ferritin molecules have never been identified, while iron-free apoferritin has been detected. An average of approximately 2500 iron (III) ions per ferritin molecule has been observed, deposited as aggregates or microcrystals of ferric hydroxide and phosphate, varying in size and shape (Chiancone et al., 1982; Finch and Huebers, 1982).

Ferritin molecules are found in almost all cell types, but is present in greatest concentration in the liver, spleen, and bone marrow. The protein exists in more than one form, with slightly different physical and chemical properties, and potentially specific
Two different ferritin subunit types appear to be responsible for the heterogeneity observed among ferritin molecules. The different subunit types differ in their amino acid sequence and molecular weight, with a heavy subunit (MW=21000) and a light subunit (MW=19000) (Chiancone et al., 1982).

1.1.5.2 Hemosiderin

As ferritin iron stores become saturated, paracrystalline structures called hemosiderin begin to form, comprised of degraded protein, iron, phosphate and hydroxide. Hemosiderin has a higher iron to protein ratio than ferritin, and as a result is not water-soluble. Therefore, hemosiderin granules can be visualized microscopically in tissue sections when stained with Prussian blue (Wixom et al., 1979). Most of the hemosiderin is found in the cells of the reticulo-endothelial system, particularly in RE cells of the spleen, bone marrow, and skeletal muscle. However, small amounts of hemosiderin are also found within parenchymal cells. As the body burden of iron increases beyond normal levels, excess hemosiderin is deposited in the liver and heart. This can reach the point that the function of these organs is impaired, and death ensues.

1.1.5.3 Reticulo-endothelial System Iron Stores

The reticulo-endothelial (RE) system is a diffuse system of cells comprising all the phagocytic cells of the body, except the circulating white blood cells. These cells phagocytose and process old and defective red blood cells, which is the RE cell source of the iron that is stored. RE cells have very few, if any, transferrin receptors on their surface, thus do not acquire and store iron delivered through transferrin.

Reticulo-endothelial cells store approximately two-thirds of the body's reserve of iron. RE cells in the liver, spleen, bone marrow and skeletal muscles are the largest RE iron stores. Within these RE cells, iron can be stored as ferritin and hemosiderin. Iron is mobilized and distributed to these cells via plasma transferrin.
1.1.5.4 Parenchymal Tissue Iron Stores

Normally, one-third of the iron stored in the body is contained within parenchymal tissues, predominantly hepatocytes. Hepatocytes have transferrin-receptor rich surfaces that bind, internalize, and store the iron delivered by monoferric and diferric serum transferrin. Therefore, the accumulation of iron into parenchymal iron stores is influenced by the concentration and saturation of serum transferrin (Cazzola and Finch, 1989).

1.1.6 Internal Iron Kinetics

1.1.6.1 Iron Exchange between Plasma and Tissues

Transferrin-bound iron is transported through the plasma to various tissues, where the iron-transferrin complexes are internalized by cells after binding to transferrin receptors on their surfaces (Figure 1.5). The transferrin receptor, a transmembrane glycoprotein dimer capable of binding two molecules of monoferric or diferric transferrin, becomes internalized after binding transferrin and fuses with an endosome in the cytoplasm. Within these endosomes, proton pumps on the endosomal membrane establish and maintain an acidic pH around 5.5, where the iron will dissociate from transferrin, but the transferrin molecule remains bound to its receptor. The iron is then released by the endosome, and ferric iron (III) is quickly reduced to ferrous iron (II), while the apotransferrin-transferrin receptor complex is shuttled back to the cell membrane. Once released by the endosome, cytoplasmic iron is destined for either heme or non-heme proteins, iron-regulatory proteins, or storage within ferritin or hemosiderin molecules. As the apotransferrin-transferrin receptor complex becomes re-exposed to the 7.35 pH of the plasma, the apotransferrin is released by the receptor, and both molecules are once again available to repeat the cycle.
Figure 1.5. Mechanism of intracellular iron transport via transferrin and the transferrin receptor (Israels and Israels, 1996).

Transferrin receptors are present on the surface of most cells. However, a relatively higher concentration of receptors is found on the surface of erythrocyte precursors, replicating cells, and hepatocytes. Focussing on the developing erythrocyte, intermediate normoblasts have the highest concentration of transferrin receptors, with approximately 800,000 receptors on their cell surface. This number progressively decreases to approximately 100,000 on the cell surface of reticulocytes, and transferrin receptors are absent from the surface of mature erythrocytes (Israels and Israels, 1996).

1.1.6.2 Pathways of Iron Exchange

Transferrin is the protein designed to pick up the iron absorbed by the gut, and deliver it to erythropoietic cells, and other metabolic and storage sites. Approximately 80% of iron exchange (between 20 – 30 mg/day) cycles through erythroid precursors in the bone marrow and macrophages. The circuit involves the phagocytosis of old and defective red blood cells by macrophages. Within macrophages, iron is removed from hemoglobin and either stored as ferritin or hemosiderin, or shuttled to the surface and transferred to serum transferrin. The circuit is completed as serum transferrin transports the iron to erythropoietic sites for heme synthesis, which is incorporated within
hemoglobin molecules of developing red blood cells. Noteworthy is the fact that macrophages have very few transferrin receptors on their surface, emphasizing that the macrophage receives very little iron from transferrin.

Hepatocytes are another key destination of plasma iron, transported either by transferrin, haptoglobin-bound hemoglobin, or hemopexin-bound heme. Hepatocytes either use this iron for metabolic processes, or store the iron as ferritin or hemosiderin.

1.1.6.3 Intracellular Iron Regulation

The regulation of intracellular iron concentration is through the control of iron uptake, and is influenced by iron storage capacity. As the intracellular functional iron concentration drops, iron is mobilized from ferritin stores and intracellular ferritin levels are reduced, while transferrin receptor synthesis is activated, and the transferrin receptor concentration on the cell surface increases. Similarly, as intracellular iron concentrations increase, ferritin molecules are synthesized and the number of transferrin receptors on the cell surface is reduced. Specialized and highly sensitive bi-functional iron-regulatory proteins (IRPs) regulate this mechanism based on the amount of iron within the cell. When intracellular iron levels are low, IRPs become unsaturated with iron, change in conformation and expose a binding site for either (1) transferrin receptor mRNA, binding to elements promoting translation, and (2) ferritin mRNA, binding to elements inhibiting ferritin translation. In the presence of elevated intracellular iron levels, IRPs remain saturated, which cannot bind to mRNA elements and influence protein production.

1.1.7 Iron Overload and Toxicity

Human beings lack the appropriate physiological mechanisms to eliminate or excrete excess iron. The body normally regulates iron balance by promoting or suppressing iron absorption, instead of through the regulation of iron excretion. Therefore, diseases associated with an increase in gastrointestinal iron absorption (ie hereditary hemochromatosis), and/or the infusion of iron through regular red blood cell transfusions, will inevitably lead to iron overload. This is a serious problem for people
with beta-thalassemia, a disease in which hemoglobin is not made normally, and is supplied by blood transfusion as needed.

1.1.7.1 Pathophysiology of Iron Overload

As iron accumulates in idiopathic or acquired iron-loading disorders, serum transferrin becomes saturated, toxic non-transferrin-bound iron (NTBI) species appear in the plasma and in tissues, and excess iron is deposited into tissue stores. Within reticulo-endothelial cells iron remains relatively harmless, however in parenchymal cells (particularly hepatocytes and myocytes) the excess iron may cause significant damage (Olivieri and Weatherall, 1999). This excess iron and NTBI catalyze reactions that generate free hydroxyl radicals, which are involved with various oxidative reactions. Structural and functional defects in the cell develop in response to this peroxidative damage to the lipid membranes of cellular organelles (O'Connell et al., 1985; Bacon and Britton, 1990). Furthermore, cell-damaging hydrolytic enzymes may be released from lysosomes as the lysosomal membrane is damaged through iron-mediated lipid peroxidation, causing further intracellular damage (Selden et al., 1980; Weir et al., 1984). The final result is cell death, pericellular necrosis and fibrosis.

Chronic iron overload is inevitable in patients who receive inadequate treatment for disorders associated with increased dietary iron absorption or receiving regular red blood cell transfusion therapy. The extent of tissue damage is dependent upon:

1. The total amount of excess iron in the body and within cells and tissues.
2. The duration of exposure to elevated iron concentrations
3. The rate of iron accumulation
4. The distribution of iron within different cells and storage compartments.
   (ie. reticulo-endothelial cell vs parenchymal cell stores)

The major tissues that are primarily affected by chronic iron overload that have been thoroughly studied include the heart, liver, and various endocrine organs.
1.1.7.2 Chronic Iron Overload and Cardiac Disease

Myocardial disease due to iron toxicity remains the most life-threatening complication of iron overload. Buja and Roberts (1971) reported that iron deposition in the heart causes hypertrophy, dilatation, degeneration of myocardial fibers, and myocardial fibrosis, and that the development of cardiac dysfunction is dependant upon the quantity of iron deposited into myocardial tissue. Wolfe et al. (1985) also reported that the extent of cardiac damage is directly related to the amount of body iron overload associated with regular transfusion therapy and inadequate, non-compliant, or unavailable iron chelation therapy in patients with beta-thalassemia.

The proposed mechanism of cardiac iron injury is that excess and non-transferrin-bound iron accumulates in the myocardium and conducting system of the heart. In iron overloaded patients, cardiac iron is initially deposited into the ventricular myocardium, while atrial myocardium usually stores much less iron and will only begin storing iron as ventricular stores become saturated (Buja and Roberts, 1971). The iron causes peroxidative damage to myocardial cell membranes, even at low concentrations, which disrupts their function and may cause fibrosis. Complications include ventricular hypertrophy, conduction disturbances, arrhythmias, and congestive failure. Unfortunately, life-threatening cardiac dysfunction becomes clinically apparent at late stages of iron-overloading disorders, often when it is too late to manage and correct, and patients often deteriorate quickly and appear to die suddenly.

1.1.7.3 Chronic Iron Overload and Liver Disease

The liver appears to be one of the first organs to reveal the toxic effects of iron overload, and the focus of the entire next section (Section 1.2). Liver abnormalities and dysfunction, including hepatomegaly, abnormal liver function tests, collagen formation, and fibrosis, is often detected within two years of initiating regular transfusion therapy (Iancu et al. 1977; Cohen. 1987; Thakerngpol et al.. 1996; Olivieri, 2001), and cirrhosis is possible within the first decade of life (Witzleben and Wyatt, 1961; Risdon, 1973; Jean et
Iron-induced liver disease eventually is life threatening, and can be aggravated by hepatitis infection and alcohol consumption.

1.1.7.4 Chronic Iron Overload and Endocrine Disease

Several clinical effects of iron overload are manifested in the second decade of life in children with iron loading disorders, often when body iron levels have accumulated to toxic levels (Section 1.2.2). Generally, after experiencing normal growth and development throughout the first decade, children with iron overload begin to experience various complications including:

- Delayed pubertal development
- Impaired sexual maturation
- Poor pubertal growth
  - Selective central hypogonadism
  - Abnormal insulin-like growth factor (IGF-1) production
  - Abnormal growth hormone secretion
- Diabetes mellitus
- Hypogonadotropic hypogonadism
- Growth hormone deficiency
- Hypothyroidism
- Hypoparathyroidism
- Adrenal androgen hyposecretion
- Zinc deficiency

The primary cause of these endocrinopathies include iron deposition and overload in the anterior pituitary, pancreas, liver, thyroid, parathyroid, and adrenal glands (Olivieri, 1999).
1.2 Hepatic Iron and the Assessment of Body Iron Levels

In this section, a brief description of liver physiology precedes a report of all the common techniques used for assessing and monitoring body iron levels. A variety of idiopathic and acquired conditions are associated with the accumulation of body iron, potentially to toxic and life-threatening levels. Excess iron is believed to induce oxidative damage, causing liver fibrosis, cirrhosis, and an increased incidence of hepatocellular carcinoma (Harford et al., 1994). Several techniques for assessing and monitoring body iron levels have been developed, but the reliability of these indicators continues to be a concern. A quantitative technique for measuring the total body iron store that is accurate, minimally-invasive, cost-effective, and readily available would be ideal.

Regular phlebotomy therapy to eliminate iron from overloaded patients also produces a direct quantitative measure of mobilisable storage iron, representative of the total body iron store. The technique described by Bothwell et al (1979) involves removing 400 ml of blood every week until the hemoglobin concentration is maintained below 10g/dL for at least two weeks (reflecting a depleted iron store and exhausted iron supply, which inhibits erythropoiesis). More recently, Angelucci et al (1997, 2000) described an alternative technique involving the removal of 6mL of blood per kilogram body weight at 14-day intervals, until normal serum ferritin concentration (<250ng/ml) and transferrin saturation (<50%) are achieved. Unfortunately, the usefulness of this technique is limited by its retrospective design, revealing the initial total body iron concentration at the completion of phlebotomy therapy (which may be several weeks or months later). Furthermore, the technique cannot be performed on patients whose health is dependent on regular transfusion therapy.

Comparatively, since the concentration of non-heme storage iron within the liver is highly reflective of the total body iron store, direct quantitative techniques that accurately evaluate the hepatic iron concentration have become the gold standard for measuring body iron levels (Bothwell et al., 1979; Angelucci et al., 2000). Two direct techniques of hepatic iron quantitation are (1) a biochemical assay of hepatic tissue (obtained through biopsy or at necropsy) and (2) magnetic susceptibility using a Superconducting Quantum Interference Device (SQUID).
1.2.1 Liver Physiology and Blood Flow

The liver is the largest internal organ of the body, and is unique among all other organs because arterial oxygenated blood, through the hepatic artery, comprises only about 25% of the total blood supply into the liver. Instead, the major blood supply is venous blood through the hepatic portal vein, which carries blood from the intestines, pancreas, and spleen. As a result, the liver is the first organ supplied and bathed with blood loaded with metabolic substances, nutrients, toxic substances absorbed from the gastrointestinal tract, and blood cells and cellular debris from the spleen. The normal function of the liver includes degrading these toxic substances, and filtering defective blood cells and cellular debris.

1.2.1.1 Structural Organization and Functional Anatomy of the Liver

The structural components of the liver include hepatocytes organized as plates or sheets of cells, connective tissue stroma, blood vessels and bile ducts that travel in the stroma, lymphatic vessels and nerves, and sinusoidal capillaries between the plates of hepatocytes. When describing the structural organization, physiology and pathology of the liver, the focus is on the acinar units of the liver, with distributing vessels of the portal triad (the portal veins, hepatic arteries, and bile ducts) along the equator between two hexagonal lobules, and hepatic venules at each pole (Figure 1.6a). The afferent vessels, the portal vein and hepatic artery, carry blood into the hepatic acinar unit, where these main vessels branch off and form a glomus of microcirculatory vessels throughout the hepatic parenchyma which empty into the sinusoids. The sinusoids, lined with a thin discontinuous endothelium, are specialized porous hepatic capillaries that bathe the hepatocytes and provide for the exchange of substances between the blood and the liver cells. Large phagocytic endocytes, the Kupffer cells, form part of the sinusoidal lining, clearing the system of particulate matter, old erythrocytes, and bacteria. The sinusoidal network of each acinar unit eventually drains into at least two terminal hepatic venules (Figure 1.6b).
The liver acinar unit can be further subdivided into 3 microcirculatory zones surrounding the afferent vascular supply vessels (ie. the hepatic artery and portal vein), representing zones supplied by blood with decreasing oxygen and nutrients (Figure 1.7).

Zone 1: Proximal to the hepatic arterioles and portal veins. The terminal hepatic arterioles join the terminal portal venules and empty into the sinusoids in this periportal zone only. Hepatic parenchymal and mesenchymal cells in this zone are bathed by blood that is relatively rich with oxygen and metabolites, and are the first to receive both nutrients and toxins in the blood. Therefore, the cells in zone 1 are the first to show morphological changes, but are the last to die if circulation is impaired. Zone 1 is further defined by specific metabolic functions, including the most active areas of protein synthesis, cytogenesis and regeneration, and gluconeogenesis.
**Zone 2:** Dividing layer of tissue between zones 1 and 3, but has no sharp boundaries. Cells in this zone have functional and morphological characteristics intermediate to those of zones 1 and 3.

**Zone 3:** Situated at the microcirculatory periphery of the acinar unit. No terminal hepatic arteriole enters this zone. The cells in this zone are furthest away from their supplying vessels, and receive blood that has already been depleted of oxygen and metabolites through the cells in zones 1 and 2. Therefore, cells in zone 3 are most sensitive to damage through anoxia, ischemia, and nutritional deficiency. However, cells in zone 3 are also the last to respond to toxic substances. Zone 3 is further defined by specific metabolic functions, including enzymatic activity of lipid, steroid, drug, and pigment metabolism, and a glycolytic liponeogenic function.

**Figure 1.7.** Microcirculatory zones in a simple liver acinus (Rappaport, 1980)
P.S. = portal space, T.h.V. = terminal hepatic venule
1.2.1.3 Hepatic Iron Metabolism and Storage

The liver is the principal organ responsible for iron detoxification and is the major site of non-erythroid plasma iron uptake and storage (only the bone marrow receives more iron from the plasma). As a result, the liver is the first organ damaged by iron overload (Hershko et al., 1972; Barry, 1974; Bothwell et al., 1979). In the liver, iron is exchanged and processed by hepatocytes (parenchymal cells of the liver), and by fixed macrophages called Kupffer cells (reticulo-endothelial cells of the liver).

The majority of the iron internalized by hepatocytes is delivered by transferrin, as hepatocytes characteristically have transferrin-receptor-rich cell surfaces. A much smaller fraction of iron is received from haptoglobin (carrying hemoglobin derived from erythrocytes which disintegrated in the circulation), catabolized, and either released back into the plasma, or stored as ferritin (Hershko et al., 1972). Serum ferritin iron is also selectively internalized by hepatocytes, resulting in a third source of iron, although normally negligible.

Reticulo-endothelial cells, including Kupffer cells, virtually lack transferrin receptors on their surfaces, and thus cannot internalize the iron bound to circulating transferrin. These cells acquire almost all of their iron through phagocytosis of red blood cells. After phagocytosis, the enzyme heme oxygenase releases iron from the porphyrin rings of hemoglobin molecules. Iron is then either released by the cell into the plasma, where it normally is accepted by transferrin, or the iron is stored within the cell as ferritin and hemosiderin.
1.2.2 Hepatic Iron Concentration and Quantitation

1.2.2.1 Hepatic Iron Thresholds of Risk of Toxicity

Interpretation of hepatic iron concentration is easiest described using Figure 1.8, illustrating the progression of iron accumulation over time. Note the values expressed in mg of iron per gram of liver tissue, dry weight, on the right axis. Normal individuals have hepatic iron concentrations approximately between 0.6 to 1.2 mg of iron per gram liver, dry weight (Brittenham et al., 1982; NEJM). Normal survival without developing any of the complications associated with iron overload, has been observed in heterozygotes for hereditary hemochromatosis, who maintain hepatic iron concentrations below 7 mg/g, dry weight (Cartwright et al., 1979; NEJM). Furthermore, from studies of homozygous hemochromatosis, exceeding the 7mg/g threshold is associated with an increased risk of iron-induced complications including hepatic fibrosis and diabetes (Niederau et al., 1985; NEJM and 1996: Gastroenterology). Elevated hepatic iron concentrations, beyond 15mg/g, have been associated with an increased risk for iron-induced cardiac disease and early death (Olivieri et al., 1992; Am J Hem).

![Figure 1.8. Template for the interpretation of hepatic iron concentration, identifying the established thresholds of risk for hemochromatosis and hemoglobinopathy patients (Olivieri, 1999).](image-url)
Of particular note is that the goal of iron-chelation therapy is to maintain liver irons below 7 mg/g, but not lower than 3.2 mg/g beyond which the issue of chelator-induced complications becomes a concern. Through body iron quantitation, iron overload can be accurately detected, and the initiation of chelation therapy can be rationally based.

1.2.2.2 Biochemical Liver Iron Quantitation Assay

The concentration of storage iron in a sample of liver tissue can be directly quantified using a simple biochemical assay. In iron overloaded patients, this measure of the hepatic iron concentration quantitatively and accurately reflects the total body storage iron concentration, particularly when the sample of liver tissue weighs greater than 1.0mg, dry weight (Barry and Sherlock, 1971; Brittenham et al., 1981; Pippard, 1989; Angelucci, 2000). This biochemical assay actually measures the total (heme and non-heme) concentration of hepatic iron, although quantifying the amount of iron in hepatic iron stores should focus on the non-heme iron content (the sum of ferritin and hemosiderin iron) of the liver. However, in the presence of iron excess, the contribution of heme-iron to the total iron content is small and insignificant (Barry and Sherlock, 1971). Therefore, complex non-heme iron extraction processes do not have to be incorporated into the assay.

The technique begins with weighing a non-fixed sample of liver tissue (usually from biopsy) to determine the ‘wet weight’ of the sample. The tissue may then be oven-dried (at 120C), and weighed to reveal the ‘dry weight’ of the sample. When the tissue sample dry weight is not recorded or reported, the wet weight of the sample can be converted to a dry weight by multiplying by a factor of 3.33, under the assumption that the liver is composed of 70% water (Brittenham et al., 1981). The wet or dry tissue is then ashed using a mixture of acids, and heated until a clear, colorless residue is obtained. A coloring agent is introduced, and the iron content is measured by comparing the 535nm atomic absorption spectrophotometry of the supernatant to a standard curve of iron concentration (Barry and Sherlock, 1971; Bothwell et al., 1979).
The accuracy and reliability of various biochemical hepatic iron quantitation assays based on the technique described above, has been well established through studies quantifying HIC in duplicate samples (Barry and Sherlock, 1971, Overmoyer et al., 1987), studies identifying the correlation between biochemical HIC and DTPA-chelatable iron \( r=0.96: \) Barry and Sherlock, 1971), and studies identifying the correlation between biochemical HIC and the amount of storage iron mobilized through phlebotomy therapy. Furthermore, Angelucci et al. (2000) revealed that the accuracy of biochemical hepatic iron quantitation is optimized when liver tissue samples exceed 1.0 mg. dry weight (approximately 3.3mg. wet weight), observing a correlation of \( r=0.98 \) between hepatic iron concentration and mobilized storage iron. Overmoyer et al. (1987) also revealed that focal lesions within the liver results in the inhomogeneous distribution of storage iron in the liver, suggesting that a sample of liver tissue may not be representative of the entire organ, and of the total body iron store.

1.2.2.3 Hepatic Iron Concentration and Liver Biopsy

Liver biopsies are performed on patients with various hemoglobinopathies receiving regular red blood cell transfusions, in order to acquire hepatic tissue for the evaluation of hepatic iron concentration (representative of body iron levels) by chemical assay, and also for histological assessment to detect and monitor the progression of liver damage. The invasiveness of percutaneous liver biopsy has been the major reason why clinicians hesitate to use liver biopsies for monitoring body iron.

1.2.2.3.1 Ultrasound-guided Percutaneous Core Liver Biopsy

The first liver biopsy on record was performed by Paul Ehrlich in 1883 (Ehrlich, 1884), and the first series was published in 1907 (Schupfer, 1907), reporting the successful acquisition of liver tissue from percutaneous biopsy. In 1958, Menghini reported a “one second” liver biopsy technique, after identifying that the rate and severity of complications could be reduced by minimizing the duration of the intrahepatic phase of the biopsy (Menghini, 1958). In 1966, with growing acceptance of Menghini’s liver
biopsy technique. Initial reports revealed that percutaneous liver biopsy could safely be performed as an outpatient procedure (Frank and Leodolter, 1966). Several clinical trials have since reported on morbidity and mortality associated with percutaneous liver biopsy (Table 1.2), investigating the safety of outpatient biopsy and the impact of ultrasound (pre-procedure or guidance) on safety and ability to acquire adequate tissue.

<table>
<thead>
<tr>
<th>No. of biopsies</th>
<th>Outpatient</th>
<th>Ultrasound Pre-procedure</th>
<th>Ultrasound Guidance</th>
<th>Mortality</th>
<th>Significant Morbidity</th>
<th>Reference</th>
<th>Dates of Biopsies</th>
</tr>
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<tr>
<td>175</td>
<td>107</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Knauer, 1978 (San Jose) Santa Clara Valley, M.C.</td>
<td>1975-76</td>
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<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.5%</td>
<td>Westaby et al., 1980 King's College H (London)</td>
<td>1977-79</td>
</tr>
<tr>
<td>1221</td>
<td>1221</td>
<td>731</td>
<td>0</td>
<td>0</td>
<td>0.3%</td>
<td>Judmaier &amp; Kathrein, 1983 (Germany)</td>
<td>1975-82</td>
</tr>
<tr>
<td>6379</td>
<td>55</td>
<td>0</td>
<td>34</td>
<td>&lt;0.1%</td>
<td>0.6%</td>
<td>Sherlock et al., 1984 Royal Free Hosp (London)</td>
<td>1974-84</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>22.5%*</td>
<td>Minuk et al., 1987 (University of Calgary)</td>
<td>1984-85</td>
</tr>
<tr>
<td>1192</td>
<td>0</td>
<td>1192</td>
<td>0</td>
<td>0</td>
<td>0.6%</td>
<td>Colombo et al., 1988 (Milano, Italy)</td>
<td>1982-85</td>
</tr>
<tr>
<td>9212</td>
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<td>0</td>
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<td>0.2%</td>
<td>McGill et al., 1990 Mayo Clinic (Rochester)</td>
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<td>0</td>
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<td>Maharaj &amp; Bhoora, 1992 (South Africa)</td>
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<tr>
<td>405</td>
<td>405</td>
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<td>0</td>
<td>0</td>
<td>3.2%</td>
<td>Janes &amp; Lindor, 1993 Mayo Clinic (Rochester)</td>
<td>1989-91</td>
</tr>
<tr>
<td>546</td>
<td>182</td>
<td>546</td>
<td>134</td>
<td>0</td>
<td>2.6%</td>
<td>Douds et al., 1995 St George's (London)</td>
<td>1989-93</td>
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<tr>
<td>1500</td>
<td>810</td>
<td>1260</td>
<td>510</td>
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<td>1.7%</td>
<td>Gilmore et al., 1995 University Hosp (Liverpool)</td>
<td>1991</td>
</tr>
<tr>
<td>222</td>
<td>222</td>
<td>222</td>
<td>0</td>
<td>0</td>
<td>0.9%</td>
<td>Smith et al., 1995 (Minneapolis)</td>
<td>1992-94</td>
</tr>
<tr>
<td>1184</td>
<td>0</td>
<td>1184</td>
<td>0</td>
<td>0</td>
<td>0.5%</td>
<td>Angelucci et al., 1995 (Pesaro, Italy)</td>
<td>1983-91</td>
</tr>
<tr>
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<td>836</td>
<td>&quot;many&quot;</td>
<td>418</td>
<td>0</td>
<td>3.2%</td>
<td>Lindor et al., 1996 Mayo Clinic (Rochester)</td>
<td>1992-94</td>
</tr>
<tr>
<td>753</td>
<td>665</td>
<td>753</td>
<td>0</td>
<td>0</td>
<td>0.5%</td>
<td>Caturelli et al., 1996 (Foggia, Italy)</td>
<td>1992-96</td>
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<tr>
<td>437</td>
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<td>300</td>
<td>0</td>
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<td>Kliewer et al., 1999 Duke U (Durham, NC)</td>
<td>1994-97</td>
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<tr>
<td>249</td>
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<td>0.4%</td>
<td>2.4%</td>
<td>Scheimann et al., 2000 Texas Children's</td>
<td>1990-97</td>
</tr>
<tr>
<td>1896</td>
<td>563</td>
<td>1167</td>
<td>458</td>
<td>0</td>
<td>0.6%</td>
<td>Cadranel et al., 2000 (France - 89 centres)</td>
<td>1997</td>
</tr>
</tbody>
</table>

*Minuk et al focussed on 7 intrahepatic and 2 subcapsular hematomas, however they included hematomas as small as 2 cm in diameter. Neither blood transfusions nor analgesia were required in any of these "complicated" cases.
1.2.2.4 Hepatic Iron Concentration and Magnetic Susceptometry

Chemical iron quantitation of hepatic tissue from biopsy remains the gold standard technique for accurately evaluating body iron levels. The only other technique that is truly compatible, with a near perfect correlation ($r=0.98$), has been magnetic susceptometry of the liver. using a Superconducting Quantum Interference Device (SQUID) (Bauman and Harris, 1967; Brittenham et al., 1982, 1988, 1993; Fischer et al., 1999). Bauman and Harris (1967) introduced this new direct and completely non-invasive magnetic technique for quantifying the iron content of the liver. However, even at the turn of the century, the lack of availability of SQUIDs globally, due to its complex and expensive design and operation, had limited its clinical use. However, new SQUID machines have recently become operational and guidelines are being developed for clinical use to begin shortly.

1.2.2.4.1 Magnetic Susceptometry

The magnetic susceptibility of any material relies upon the magnetic behavior of the material when exposed to a constant magnetic field. In a constant magnetic environment, all materials acquire their own complimentary constant magnetic field, varying in magnitude and direction based on the composition of the material. Ferromagnetic materials (eg. bar magnet) acquire strong fields oriented in the same direction as the applied field. At the opposite end of the spectrum, diamagnetic materials acquire weak complimentary fields in the opposite direction of the applied field. Paramagnetic responses are the intermediate responses between ferromagnetic and diamagnetic, oriented in the same direction as the applied field, but a fraction of the magnitude. Most human biological materials are diamagnetic. Although none are ferromagnetic, the iron contained within hepatic ferritin and hemosiderin contributes to a liver that is paramagnetic, with ferromagnetic iron contained within diamagnetic tissue. Any other surrounding paramagnetic materials and diamagnetic tissues has negligible effect. As a result, the acquired magnetic field of the liver is directly proportional to the number of iron atoms contained within the hepatic storage compartments.
1.2.2.4.2 Superconducting Quantum Interference Device (SQUID)

A SQUID is a CT scanner sized device designed and developed for magnetic susceptometry (Figure 1.9). The principle component is a magnetic susceptibility superconducting transducer probe, containing a material that creates a constant magnetic field (either a permanent magnet or a current carrying coil), and an extremely sensitive magnetic field detector. The function and extreme sensitivity of a SQUID is attributable to three quantum-mechanical properties, (1) zero resistance of a supercooled superconductor, (2) voltage created across Josephson junctions in a superconducting material, and (3) flux quantization of a superconducting loop.

![Figure 1.9. A Superconducting Quantum Interference Device (SQUID), which uses magnetic susceptometry to quantify the amount of iron within a person's liver (Brittenham, 1988).](image)

In ordinary conductors, single electrons are deflected by impurities, defects, and vibrations along the lattice structure of the material, resulting in resistance. In a superconducting material, two electrons of opposite spin and momentum bind together to form a Cooper pair, acquiring no net spin and momentum (Clarke, 1994). In superconductors, the material is supercooled in order to minimize lattice vibration and the energy binding a Cooper pair of electrons is strong enough to prevent scattering and separation. Therefore, the Cooper pairs travel through the superconductor without resistance. The superconducting material used in first generation SQUIDs needed to be
maintained at near absolute zero temperatures, using a 4.2K (-269°C) bath of liquid helium.

In a SQUID, the superconducting loop is transformed into the most sensitive magnetic field detector known, capable of resolving a relatively minute change in magnetic field within a large background field, by incorporating Josephson junctions into its structure. A Josephson junction is a very thin insulating barrier separating two superconductors, which acts as a barrier to current. The junction must be microscopically thin so that a proportion of Cooper pairs can still cross the barrier without breaking up; thus the junction acts as a relatively weak superconductor. However, the junction generates enough resistance to create a voltage. The maximum current that can flow across a Josephson junction is called the critical current of the circuit.

The magnetic field detector of a SQUID consists of a superconducting ring with two Josephson junctions (Figure 1.10). As the critical current flows around the ring, it generates a magnetic flux (defined as the product of the magnetic field and the area enclosed by the ring). The period of these oscillations defines the flux quantum. Any external magnetic field alters the flow of electrons through the ring, disrupting the critical current of the SQUID, and changing the quantum phase difference across each of the two junctions. The SQUID converts the tiniest change in magnetic flux into a measurable voltage. The Cooper pairs of electrons travelling through a superconducting loop with Josephson junctions generate a magnetic flux. These electrons are extremely sensitive to an external magnetic field, which alters the current and magnetic flux of the loop.

Figure 1.10.
SQUID superconducting ring with Josephson junctions (Clarke, 1994).
Specially designed SQUID machines have been developed for making hepatic iron measurements in humans, particularly in those with iron overload and other disorders in iron metabolism (i.e., hemochromatosis and thalassemia). Due to the magnetic sensitivity of the SQUID transducers, the devices have been built in facilities designed for magnetic sensitivity. The patient lies on their back directly underneath the transducer, which is positioned over the patient's liver. With the SQUID actively producing a constant magnetic field, the patient is gradually lowered away from the transducer, which detects the weakening magnetic influence of the hepatic iron that is directly proportional to the concentration of iron in the liver. An expanding water bellows occupies the space between the transducer and the patient's body. The magnetic susceptibility of the water in the bellows cancels out that of the subcutaneous tissues overlying the liver, and instrumental noise is considered negligible for a superconducting material. The result is the accurate quantitation of hepatic iron concentration.

1.2.2.4.3 Limitations of SQUID Hepatic Iron Quantitation

Implanted metal devices contaminate the magnetic fields generated during SQUID hepatic iron quantitation (Brittenham et al., 1982). These include surgical clips, braces, steel plates, artificial joints, and pacemakers.

The worldwide lack of availability of SQUIDs is the greatest limiting factor to its widespread clinical use for iron quantitation. Construction and operation of a SQUID has had enormous costs, including the more than $1,000,000 per machine cost of construction of the SQUID and its facility (Brittenham et al., 2001), which must be insulated from external magnetic influences. The purchase and handling of the liquid helium, required to maintain the near absolute zero temperature of the superconductor, is not only very expensive but also requires physical and bioengineering expertise.

Although not formally documented, it is also believed that SQUID hepatic iron quantitation may not be accurate in patients who are obese, due to an inappropriate amount of subcutaneous tissue overlying the liver, which may contaminate the magnetic field of the hepatic iron.
Hepatic iron quantitation through magnetic susceptometry has been proven to be as reliable as iron quantitation through the biochemical assay of liver biopsy tissue weighing greater than 1 mg, dry tissue (Brittenham et al., 1982, 1988 and 1993; Fischer et al., 1999). However, unlike liver biopsy, susceptometry cannot provide any information on the histology of the liver, or the progression of liver disease.

1.2.2.4.4 New Technology and 2nd Generation SQUID Machines

Recent technological advances are providing the means of decreasing both the cost and complexity of SQUID magnetic susceptometry, including the development of superconductors functioning at more manageable temperatures operating in liquid nitrogen around 77°K (-196°C). This is contributing to the development of new SQUID machines that promise to be more affordable, and eventually readily available for clinical use. Attention has also been focussed on highly sensitive magnetometers that operate at room temperature, and their potential to replace the superconducting loop of a SQUID susceptibility probe (Brittenham et al., 2001).

1.2.2.5 Indirect Techniques for Hepatic Iron Quantitation

Computed topography and magnetic resonance imaging have been used to assess hepatic iron concentration. Currently, both modalities are neither clinically available nor reliable for quantitative interpretation, especially in patients with elevated body iron stores (Mitnick et al., 1981; Guyader et al., 1989; Houang et al., 1979; Olivieri et al., 1989; Stark et al., 1985; Johnston et al, 1989; Kaltwasser et al., 1990; Bonkovsky et al., 1990; Gomori et al., 1991; Liu et al., 1993; Jensen et al., 1994; Jensen et al., 1995).

1.2.2.5.1 Computed Topography (CT)

Computed topography has been suggested as a non-invasive direct technique for estimating hepatic iron concentration. Iron overloaded livers can be identified or recognized using CT, due to an increase in the attenuation of the liver when iron-loaded.
Both single-energy and dual-energy computed topography, however, proved to incapable of adequately resolving differences in hepatic iron concentration, and could reliably identify only massive differences in hepatic iron concentration (Mitnick et al., 1981). Furthermore, fatty changes in the liver are also visualized by CT, which contaminate the interpretation of hepatic iron concentration. Several studies have thus concluded that CT is inappropriate and unreliable for the measurement of iron overload (Mitnick et al., 1981; Guyader et al., 1989; Bonkovsky et al., 1990).

1.2.2.5.2 Magnetic Resonance Imaging (MRI)

In the 1980s, magnetic resonance imaging became a very promising candidate for non-invasive hepatic iron quantitation. The technique is considered to provide an indirect measure of hepatic iron concentration, by quantifying the effect of ferritin- and hemosiderin-iron on the proton resonance behavior of tissue water (Brittenham, 2001). However, this interaction is poorly understood. Nevertheless, an increase in hepatic iron concentration results in a decrease in signal intensity, which is visualized in MR images as a darker appearance of the liver. Standard spin-echo MR imaging, however, has been proven to be incapable of distinguishing mild from marked levels of iron overload. Recently, a newer MRI technique using gradient-recalled-echo (GRE) sequences, which is more sensitive to the field inhomogeneities produced by paramagnetic tissue-iron, has proven to be more accurate at estimating hepatic iron concentration than spin-echo MR (Bonkovsky et al., 1999). However, the variability in signal intensity at elevated hepatic iron levels (greater than 9 mgFe/g liver, dry weight) is still very broad, limiting the clinical usefulness of this technique in more severe cases of iron overload (when we would be most concerned about the reliability of these measures) (Brittenham, 2001). The greatest challenges of hepatic iron quantitation using MR continue to be poor resolution at elevated hepatic iron concentrations, the artifact created from motion, even when using shorter imaging sequences, and the lack of standardized MR imaging (interinstrumental variability), described as the variation in absolute signal intensity observed between different machines (Stark et al., 1985; Johnston et al.1989; Kaltwasser
et al., 1990; Bonkovsky et al., 1990; Gomori et al., 1991; Liu et al., 1993; Jensen et al., 1994; Jensen et al., 1995).

1.2.3 Indirect Assessment of Body Iron Levels

Indirect measures of body iron focus on physiological changes influenced by body iron levels, instead of measuring the actual amount of iron in tissue and body iron stores. Although indirect methods, blood tests or imaging techniques, are undoubtedly less invasive than a liver biopsy, they are also influenced by factors other than body iron levels, limiting their reliability. In order to assess the value of various indirect techniques for determining the amount of storage iron in iron overloaded patients, it is necessary to examine the relationship between each of these indicators and direct quantitative measures of iron stores, in normal subjects and in those with iron overload.

1.2.3.1 Serological Markers

1.2.3.1.1 Serum Ferritin Concentration

Serum ferritin concentrations are measured on venous blood samples collected by venipuncture, and measured using an immunoradiometric sandwich assay. The normal range is between 14 and 400 ng/ml of blood. It has been well established that patients with idiopathic hemochromatosis acquire elevated serum ferritin levels (Prieto et al., 1975; Edwards et al., 1977; Halliday et al., 1977; Edwards et al., 1980). Elevated serum ferritin levels are also observed in transfusional iron overload (Jacobs et al., 1972; Lipschitz et al., 1974; Letsky et al., 1974; Propper et al., 1977; Hoffbrand et al., 1979). However, several reports have revealed that serum ferritin levels do not quantitatively reflect body iron levels, and are thus limited in their clinical usefulness (Walters et al., 1973; Birgegard et al., 1977; Jacob et al., 1980; De Virgillis et al., 1980; Worwood et al., 1980).

In normal human subjects, serum ferritin concentration correlated poorly with body iron levels measured through quantitative phlebotomy (Walters et al., 1973;
Birgegard et al., 1977; Jacob et al., 1980). In patients with a range of body iron concentration, from deficient to very elevated, a correlation of $r=0.59$ was reported (Brittenham et al., 1981). In adult patients recently cured of thalassemia though bone marrow transplantation, phlebotomy therapy to eliminate pre-existing iron overload revealed a correlation of $r=0.67$ between initial SFC (pre-phlebotomy therapy) and the total body iron store (mobilized storage iron) (Angelucci et al., 2000). In children with thalassemia, De Virgillis et al. (1980) reported no correlation between serum ferritin concentration and both the numbers of transfusions ($r=0.176$. p-value reported as not significant) and the hepatic iron concentration ($r=0.159$. p-value reported as not significant). Worwood et al (1980) reported a correlation between SFC and the volume of blood transfused of only $r=0.38$ among regularly transfused thalassemia major patients. Other, more recent, studies have reported serum ferritin correlation with hepatic iron concentration ranging between 0.69 to 0.76 in patients with B-thalassemia (Brittenham et al., 1993; Nielsen et al., 1995; Piga et al., 1999). However, stronger correlations as high as $r=0.96$ have also been reported in patients with thalassemia (Prieto et al., 1975; Aldouri et al., 1987).

Although Brittenham et al. (1993) also reported a correlation of $r=0.75$ between SFC and HIC in Sickle Cell Disease, several other investigators have reported much weaker and often insignificant correlations, ranging between $r=0.04$ to $r=0.35$ (Vieira et al., 1999; Harmatz et al., 2000). Also noteworthy is that these groups, along with others, have also reported very weak correlations between SFC and both the duration of regular transfusion therapy ($r=0.308$: Harmatz et al., 2000) and the total volume of blood transfused ($r=0.05$: Vieira et al., 1999; Hussain et al., 1978; Adekile et al., 1985). Altogether, these observations strongly suggest that serum ferritin may be an inaccurate and inappropriate marker for iron overload in sickle cell disease.

A serum ferritin:aspartate transaminase ratio has also been suggested as an indicator of body iron burden (Prieto et al., 1975), however a limited correlation to body iron load, similar to that observed with serum ferritin alone, was observed in patients with hemochromatosis and thalassemia major. (Valberg et al., 1978; Worwood et al., 1980).
SFC may fluctuate independently of body iron stores due to inflammation, infection, ascorbate deficiency, neoplasia, malnutrition, liver disease, or various other disorders (Brittenham et al., 1981; Gordeuk et al., 1987).

1.2.3.1.2 Serum Ferritin Iron Concentration

In 1995, Herbert et al initially described a new assay capable of measuring the amount of iron contained within serum ferritin (Herbert et al., 1995; Herbert et al., 1997). The authors proposed that serum ferritin iron concentration is more reflective of body iron concentrations than serum ferritin protein concentration, making it a more appropriate marker or indicator for monitoring iron accumulation and overload.

The authors hypothesize that during an acute phase inflammatory response, while serum ferritin protein concentrations may escalate, the total amount of iron contained within all circulating serum ferritin remains constant. Instead, the iron may redistribute itself among the increased number of circulating ferritin molecules, resulting in a decreased iron saturation of serum ferritin. The iron saturation of serum ferritin has been shown to decrease in response to an acute-phase response (ten Kate et al. 1997).

Further investigation of serum ferritin iron concentration as a potential indicator of body iron levels has not been encouraging. In patients with hemochromatosis and thalassemia, recently reported correlation between body iron stores and serum ferritin iron concentration, r=0.63 (p<0.001), are no better than correlation with serum ferritin protein concentration (Nielsen et al., 2000). However, these results may be interpreted as useful in clarifying the influence of factors other than inflammation on fluctuations in the serum ferritin concentration.

1.2.3.1.3 Serum Transferrin Concentration

The concentration of transferrin protein circulating in the plasma/serum has also been suggested as an indicator of body iron levels. The liver is likely the only source of serum transferrin, and the production of transferrin appears to be inversely proportional to hepatocyte ferritin concentration (Morton and Tavill, 1977). However, while
decreased serum transferrin concentrations have been reported in iron overloaded patients, no reliable quantitative relationship has been observed.

1.2.3.1.4 Iron-Binding Capacity, Serum Iron, and Transferrin Saturation

Serum iron is the concentration of transferrin-bound iron present within the serum, and normally it is between 50 to 150 µgFe/dL of blood (9.0 – 27.0 µmol/L). Once the serum transferrin and serum iron concentration are revealed, the transferrin saturation is defined as the molar ratio of:

\[
\frac{\text{serum iron concentration}}{\text{serum transferrin concentration}} \times 100\%
\]

Transferrin is normally only about 1/3 saturated with iron, carrying 100 µgFe/dL (20 µmol/L) of blood (=serum iron). Therefore, there is a substantial unsaturated plasma iron binding capacity. This iron binding capacity of all iron ligands within the serum, accounted for almost entirely by transferrin, is also of clinical interest. There are three components involved in defining the iron binding capacity of serum:

(1) Serum iron concentration
(2) Total iron binding capacity (TIBC), which is the maximum amount of iron that can be bound. Normally this is between 270 to 540 µgFe/dL of blood (48.0 – 96.0 µmol/L) in adults, and between 150 to 450 µg/dL (26.0 – 78.0 µmol/L) in children.
(3) The unsaturated iron binding capacity (UIBC) is the difference between the TIBC and the serum iron. It is normally about 200 micrograms per 100 milliliters.

Iron binding capacity is used in the differential diagnosis of certain diseases. In conditions associated with increased need for iron (iron deficiency or late pregnancy), TIBC is increased, but saturation is decreased from the normal 33%. In hemochromatosis, TIBC is low, but it is saturated. Certain other clinical conditions are associated with their own characteristic patterns of TIBC and percent saturation (Figure 1.11).
Total Iron Binding Capacity (TIBC) = Unsaturated Iron Binding Capacity (UIBC) + Serum Iron (SI)

**Figure 1.11.** (a) Interpretation of the serum iron and iron binding capacity, and (b) the serum iron and iron binding capacities in various physiological and pathological conditions (Baggott and Dennis, 1995).

While both elevated plasma iron concentrations and increased transferrin saturation suggest iron overload, no quantitative relationship has been defined. Furthermore, several other factors may also influence these levels, including inflammation, infection, neoplasia, malnutrition, and liver disease, making these measures unreliable indicators of body iron levels (Brittenham et al., 1981).

### 1.2.3.1.5 Serum Transferrin Receptor Concentration

Transferrin receptors, mainly from the surface of cells within the erythroid marrow, are proteolytically cleaved from cell membranes and released into the circulating plasma as red cells mature (Huebers et al., 1990; Cook, 1999; Brittenham, 2001). In conditions of erythroid hyperplasia, known to be associated with increased iron absorption, the serum transferrin receptor concentration increases. Therefore, serum transferrin receptor concentration could be considered an indirect indicator of erythroid
activity, which may be useful in predicting iron overload in conditions of ineffective erythropoiesis. Immunoassays capable of measuring the serum transferrin receptor concentration have recently been developed. The normal reference range is 0.65 – 2.1 mg/l, and children usually have higher concentrations than adults (Virtanen et al., 1999).

The accuracy and reliability of the serum transferrin receptor concentration as an indicator of body iron overload has not been appropriately established. Khumalo et al. (1998) reported a decrease in serum transferrin receptor concentration in the presence of iron overload, while Cazzola et al. (1999) suggested that elevated concentrations indicate iron overload. Cook et al. (1994) also realized that the erythroid hyperplasia associated with sickle cell disease, is responsible for increased serum transferrin receptor concentrations, contaminating any relationship to body iron levels. In summary, no quantitative relationship has been described between the serum transferrin receptor concentration and the amount of iron in the body. Therefore, serum transferrin receptor concentration does not appear to be a useful indicator of iron overload.

1.2.3.1.6 Non-Transferrin-bound Plasma Iron (NTBPI)

In various conditions including iron overload, the transferrin iron binding capacity may be exhausted and non-transferrin bound iron species may be detected in the plasma (Hershko et al., 1998). Assays capable of measuring the amount of NTBPI may be useful for detecting dangerous levels of iron overload and indicating an increased risk of iron-related complications (Porter et al., 1996). However, no quantitative relationship to the body iron concentration has been reported.

1.2.3.2 Imaging Techniques (non-hepatic)

1.2.3.2.1 Magnetic Resonance Imaging

MR imaging has proven to be capable of visualizing the degree of iron overload within the heart; a low signal intensity reflecting an elevated cardiac iron concentration (Olivieri et al., 1992; Liu et al., 1996; Olivieri and Brittenham, 1997). Periodic cardiac MRIs were able to identify changes in cardiac iron concentration that corresponded to
changes in body iron in response to iron chelation therapy. However, a reliable quantitative relationship capable of revealing the body iron concentration from cardiac MRI has never been established.

MR imaging of the anterior pituitary has also been investigated for its potential to identify conditions of increased risk for iron-related endocrinopathies, and to reveal the degree of body iron burden (Fujisawa et al., 1988; Berkovitch et al., 1993). Signal intensity of the anterior pituitary appeared to be moderately well correlated with the pituitary iron concentration, but could not offer a reliable quantitative measure of body iron equivalent to biochemical or susceptometric hepatic iron quantitation.
### 1.3 Iron Chelation Therapy

The accumulation of excess iron is inevitable in patients receiving regular red cell transfusion therapy, as every milliliter of transfused red cells introduces another milligram of iron into the body, and humans lack appropriate physiological mechanisms to regulate iron excretion and eliminate excess iron. The only possible treatment for iron overload in transfusion-dependant patients is the mobilization and removal of excess iron from the body using chelating agents.

#### 1.3.1 Principles of Iron Chelation Therapy

The goal of iron chelation therapy is to eliminate enough iron from the body to balance the amount of iron being introduced through transfusions, and/or to maintain safe body iron levels, not associated with an increased risk of iron-related complications. The characteristics of an ideal iron chelator would include:

1. It must have a high affinity for iron, not affecting the excretion of other ions or molecules.
2. It must selectively target non-functional iron ligands (i.e., storage iron not hemoglobin iron).
3. It must be able to penetrate into tissues and cells to access iron from stores.
4. It must persist (avoid being cleared or metabolized) in the body long enough to reach, mobilize, and bind to iron.
5. It must form a stable water-soluble iron complex, which can be easily excreted.
6. It must be non-toxic, particularly at high doses.
7. It must be easily and affordably synthesized.

#### 1.3.2 Deferoxamine

In the early 1960s, several reports identified the first two iron chelators that proved to have some success at removing iron from overloaded patients with
hemochromatosis and thalassemia; deferoxamine (DFO) and diethylenetriaminepentaacetic acid (DTPA) (Bickel et al., 1960; Fahey et al., 1961; Bannerman et al., 1962; Smith and Sephton, 1962; Heilmeyer and Wohler, 1963; Muller-Eberhard et al., 1963; Smith, 1965; Keberle, 1965). DFO eventually became the chelator of choice, as DTPA interfered with magnesium metabolism, had to be administered by deep intramuscular injection and had to be mixed with procaine, which caused toxic reactions.

1.3.2.1 Structure and Mechanism of Action

Deferoxamine (DFO) is a complex hexadentate hydroxylamine (sideramine) produced by the fungus *Streptomyces pilosus*. It is a large (MW 656.8) straight-chained molecule composed of one molecule of acetic acid, two molecules of succinic acid, and 3 molecules of 1-amino-5-hydroxylaminopentane. As a molecule of DFO encounters a ferric ion, the DFO molecule wraps around the ion, forming a 1:1 DFO:iron complex. The three hydroxamic acid groups interact with the ferric ion, surrounding it in an organic shell, creating a very stable iron complex (stability constant of $10^{31}$) (Will, 1999).

![Deferoxamine molecule](image)

**Figure 1.12.** (a) Deferoxamine straight-chain molecule and (b) Ferrioxamine (DFO wrapped around ferric ion) (Modell and Berdoukas, 1984).
In vitro and incubation studies have revealed that:

1. DFO does not extract porphyrin iron (ie. from hemoglobin)
2. DFO can remove iron stored within ferritin and hemosiderin
3. DFO can, to a lesser extent, withdraw iron from transferrin.
   (ie. removes 10-15% of iron when exposed to totally saturated transferrin).
4. DFO is rapidly broken down in the plasma (Keberle. 1965).
5. DFO protects myocardial cells from the effects of iron overload (Hershko et al, 1984).
6. DFO reverses iron damage in iron-overloaded cells (Hershko et al., 1984)

Animal studies have also revealed that:

1. DFO does not affect the excretion of sodium, potassium, calcium, magnesium, manganese, zinc, cobalt, nickel, and copper.
2. DFO can penetrate into tissues and cells to access iron stores.
3. DFO is excreted in the bile and in the urine.
4. Daily doses of 50mg/kg resulted in a 5-fold increase in iron excretion from normal rabbits, and a 16-fold increase from iron overloaded rabbits (Keberle. 1965).

Iron released from red cell breakdown by macrophages is the major source of chelatable iron in the plasma (Porter, 2001). As the transferrin saturation increases in response to regular transfusions, the size of this chelatable iron pool increases. As a result, defereroxamine metabolism actually decreases, as fewer unbound DFO molecules reach the liver, where they can be quickly metabolized by oxidative deamination (Porter, 2001). DFO can also penetrate into the parenchymal and reticulo-endothelial cells of tissues, notably the liver, and access the iron stored within ferritin and hemosiderin. Ferrioxamine (DFO bound to iron) is then excreted in the urine and feces (bile from the liver) (Pippard, 1989, Porter, 2001).
Brittenham et al (1994) and Olivieri et al (1994) were the first to report the independent effect of deferoxamine in decreasing the risk of death in patients with iron overload associated with regular transfusion therapy. These studies along with others have also reported that adequate DFO therapy can:

1. Prevent the accumulation of iron to toxic levels.
2. Achieve a net negative iron balance reducing iron overload (Brittenham et al., 1994).
3. Prevent cardiac disease and early death (Brittenham et al., 1994).
4. Arrest the progression of hepatic fibrosis, and development of cirrhosis (Barry et al., 1974).
5. Prevent various endocrine disorders, including thyroid, parathyroid, adrenal abnormalities, and abnormal sexual maturation (Bronspiegel-Weintrob et al., 1990)

Based on the 1:1 binding ratio, if perfectly efficient, 1 gram of DFO would remove 85 milligrams of ferric iron. However, DFO is not an ideal iron chelator. It is quickly cleared from the plasma by the kidneys (t$_{1/2}$ = 5 - 10min), and it is also metabolized by the liver and excreted in the bile, limiting the amount of time it has to reach, liberate, and bind to iron (Keberle, 1964; Summers et al., 1979; Will, 1999). Bolus IV doses are ineffective as most of the DFO is quickly cleared through the kidneys, before the drug has a chance to chelate significant amounts of iron. Therefore, large doses administered continuously over extended periods of time are more effective and capable of mobilizing iron at a rate equivalent to, or exceeding the rate of transfusional iron accumulation (Modell and Berdoukas, 1984). Secondly, DFO is a large molecule, and is poorly absorbed by the GI tract. As a result, DFO cannot be administered orally, and instead must be administered parenterally. Finally, the cost to produce DFO is unfortunately expensive, seriously limiting worldwide availability.
1.3.2.2 Administration and Compliance

The ability of DFO to chelate and remove iron from the body became evident in the early 1960s, following trials using intramuscular, intravenous, and/or subcutaneous bolus administration (Bannerman et al., 1962; Sephton-Smith, 1962). Since then, 24-hour intravenous and subcutaneous constant infusions of DFO proved to be very effective at eliminating iron, particularly in very overloaded patients (Modell and Beck, 1974; Propper et al., 1976; Hussain et al., 1976; Propper et al., 1977). Patients treated with IV-DFO have shown a significant improvement in cardiac health (echocardiographic changes) compared with those treated with only SC-DFO (Di Gregorio et al., 1987). However, 8-12 hour nightly subcutaneous infusions of DFO, performed at home using ambulatory pumps, has proven to be effective at controlling body iron levels, relatively convenient to use clinically, and reasonably well-tolerated by patients, and has become the standard technique of chelation therapy, wherever affordable and available (Olivieri and Brittenham, 1997). Wolfe et al (1985) confirmed that compliance with standard subcutaneous deferoxamine therapy protects against the development of cardiac disease in regularly transfused beta-thalassemia patients. Unfortunately, chelation therapy often becomes critical as the patients reach their teenage years, a period of life associated with rebelliousness and feelings of invulnerability, and these patients tend to be unreceptive to the treatment. In fact, it has been suggested that non-compliance with chelation therapy has become the underlying cause of death among thalassemia patients.

Nevertheless, the undisputed conclusion of several key studies focussing on survival in regularly-transfused patients, is that those compliant with regular subcutaneous deferoxamine therapy are surviving significantly longer than those not compliant or not receiving SC-DFO chelation therapy. Appropriately chelated patients have begun experiencing iron-related disease free survival beyond the fourth decade of life.
1.3.2.2.1 Ascorbic acid (Vitamin C) Supplementation

The increased metabolism of ascorbate causing ascorbate deficiency has been identified in thalassemia patients with iron overload (O'Brien, 1974; Chapman et al., 1982). Ascorbic acid appears to have the ability to solubilize iron into a chelatable form accessible to deferoxamine (Hussain, 1977; Bridges and Hoffman, 1986). Pippard et al (1982) observed that in iron overloaded patients, ascorbic acid supplementation during chelation therapy significantly increases the amount of excreted iron. However, ascorbic acid supplementation would be detrimental if patients are not being chelated, as the iron that is liberated will not be removed from the body, and instead the concentration of toxic iron species in the plasma would increase.

1.3.2.3 Dosage and Toxicity

A tremendous advantage of DFO chelation therapy is its lack of toxicity in iron loaded patients, during both short- and long-term therapy, and this chelator remains the only iron chelator widely available for clinical use for the treatment of iron overload. The toxic dose in humans has yet to be established. Clinical doses usually range between 20 - 50 mg/kg/day. Toxic effects have been observed using doses exceeding 50mg/kg/day, while lower doses are also toxic among patients who are not excessively iron overloaded (Porter and Huehns, 1989). Therefore, the goal of chelation therapy must not be to achieve normal hepatic iron concentrations (below 1.2 mg/g,dw). aiming instead to maintain liver irons between 3.2 to 7mg/g,dw (Brittenham et al., 1982; Olivieri et al., 1999).

Toxic effects of excessive deferoxamine therapy include ocular and auditory abnormalities (Olivieri et al., 1986; Porter and Huehns, 1989; Borgna-Pignatti et al., 1984; Orton et al., 1985; Rahi et al., 1986; Dickerhoff, 1987), sensorimotor neuropathy (Giardina et al., 1993), renal dysfunction (Koren et al., 1992), and pulmonary dysfunction (Freedman et al., 1990), which have been observed when doses exceed 50 mg/kg/day,
and in patients with only mildly elevated hepatic iron concentrations (usually below 3.2 mg/g,dw). It has recently been recommended that DFO doses not exceed 50mg/kg/day (Olivieri and Brittenham, 1997). With the appropriate monitoring of body iron levels to guide prescription and avoid the toxicity of excessive iron chelation, DFO therapy has proven to be able to reduce iron overloaded body iron concentrations and maintain iron levels within safe ranges (between 3.2 - 7 mg/g, dry weight) (Olivieri, 1999).

1.3.2.4 Cost and Global Use

Despite the potential benefits of DFO chelation therapy, and evidence of established programs of therapy successfully controlling iron overload in regularly transfused patients, the greatest limitation to worldwide use of DFO continues to be its high cost of production and administration. These costs include not only the purchase of the drug (estimates of approximately US$500.00/month), but also must include the costs of infusion pumps (estimates of approximately US$200.00) and other supplies (Vazirani, 2000). Unfortunately, most of the world’s hemoglobinopathy patients live in developing countries without the resources to support an iron chelation program.

1.3.3 Deferiprone

In an effort to improve patient acceptance and compliance with chelation therapy, and reduce the cost of treatment, the first generation of orally active iron chelators were developed, with the greatest focus on 1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone or L1). Deferiprone was patented and introduced in 1982 as a potential alternative iron chelator to deferoxamine for managing iron overload (Hider, 1982). Deferiprone was initially reported to have beneficial short-term effects on iron balance, and was also associated with an improvement in patient compliance with chelation therapy (Olivieri et al., 1995). However, long-term studies identified an eventual stabilization or increase in hepatic iron concentration in deferiprone-chelated patients, as well as other possible deleterious effects including the progression of hepatic fibrosis, neutropenia, and agranulocytosis (Hoffbrand et al., 1998; Olivieri et al., 1998).
1.3.3.1 Structure and Mechanism of Action

Deferiprone is a neutral, bidentate iron chelator (Figure 1.13), which forms a 3:1 L1:ferric iron complex (3 molecules of L1 are required to bind to the six coordination sites of 1 ferric iron molecule) at the physiological pH of 7.4 (Hoffbrand et al., 1998; Hershko et al., 1998). Due to its relatively small size (MW: 139.2), deferiprone is easily and rapidly absorbed through the GI tract and appears in the blood within minutes (Kontoghiorghes et al., 1990). In a stepwise sequence, three molecules of L1 eventually surround an iron molecule, and the complex is excreted mainly in the urine (Olivieri and Brittenham, 1997). Transferrin has been identified as a source of iron chelatable using L1 (Turcot et al., 2000). However, due to its neutral charge, the drug also appears to be able to permeate cell membranes and access intracellular iron stores, removing iron from ferritin and hemosiderin (Kontoghiorghes, 1986; Kontoghiorghes, 1986; Kontoghiorghes et al., 1987; Hoffbrand, 1995; Hershko et al., 1998). While animal studies using rodents and rabbits have revealed variable efficacy of L1 therapy, studies in iron loaded primates have reported that L1 was incapable of clearing enough iron from the body to achieve net iron balance (Bergeron et al. 1992). Although, undisputedly less efficient at clearing iron from the body compared to DFO, the greater the body iron load, the more effective the chelator is at mobilizing iron (Kontoghiorghes et al., 1987).

![Molecular structure of 1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone, L1)](image-url)
1.3.3.2 Administration and Compliance

L1 is an oral iron chelator, which appears to be most effective when administered in large divided doses (usually three doses per day), suggesting that constant serum levels of the chelator can be established and maintained throughout the day. Patients are eagerly anticipating a safe and effective oral chelation therapy, which would be a welcome alternative to the current standard subcutaneous 8 – 12-hour nightly infusion of DFO. Undoubtedly, compliance with therapy would improve if chelation could be administered orally.

1.3.3.3 Dosage and Toxicity

Of the limited number of studies investigating the efficacy of deferiprone, several have used a dose of 75 mg/kg/day in 2-4 divided doses. In some patients doses up to 120 mg/kg/day were administered while in others lower doses of 50 mg/kg/day were believed to be sufficient. While deferiprone at a dose of 75mg/kg/day results in a urinary iron excretion equivalent to DFO at a dose between 30 – 40 mg/kg/day, fecal iron excretion with deferiprone is much less than that of DFO at these doses. Therefore, L1 has been identified as a less efficient and effective iron chelator (Olivieri et al., 1990; Brittenham et al., 1992; Collins, 1994).

Various toxicities reported from animal studies include:

- anemia
- leukopenia
- thrombocytopenia
- pancytopenia
- bone marrow atrophy
- gonadal atrophy
- thymic atrophy
- adrenal hypertrophy
- growth retardation
- embryotoxicity

(Porter et al., 1989; Porter et al., 1990; Porter et al., 1991; Berdoukas et al., 1993)
In a gerbil model of human iron overload, hepatic and cardiac fibrosis was identified in animals treated with a structurally similar hydroxypyridone (Carthew, 1994).

Human trials have also reported L1 toxicities including musculoskeletal pain and arthralgias (35%), severe and recurrent neutropenia (12%), agranulocytosis (1-2%), dermatologic changes (1%), nausea (20%), and liver dysfunction, even using common clinical doses of 75 mg/kg/day (Hoffbrand et al., 1989; al-Refaie et al., 1992; Agarwal et al., 1992; Berkovitch et al., 1994; al-Refaie et al., 1994; al-Refaie et al., 1995; Hoffbrand, 1996). Furthermore, the potential adverse immunological effects of L1 have been suspected of possibly contributing to several deaths related to infections and immune dysfunction among L1-treated patients (Mehta et al., 1993; Olivieri and Brittenham, 1997).

1.3.3.4 Cost and Global Use

Due to the uncertainties about the efficacy and concerns of the toxicity of deferiprone, the oral chelator has not been approved for clinical use in several countries, including Canada and the US. Despite these concerns, L1 is becoming more widely available, due to its lower cost and increased patient compliance, particularly in Asian and Middle Eastern countries where patients are often inadequately chelated, due to poor socio-economic status. In these countries, it has been suggested that deferiprone therapy can be supplied at an eighth of the cost of DFO (Kontoghiorghes et al., 2000). In India, the monthly cost of L1 is estimated between US$60.00 – US$100.00, compared to a monthly DFO cost of over US$500.00, not including the approximately US$225.00 cost of the pump for DFO infusion (Vazirani, 2000). Nevertheless, the expanding clinical use of L1 appears to be premature, as several concerns have yet to be resolved.
1.4 Pathophysiology of Thalassemia

Thomas Cooley and Pearl Lee have been credited as the first to describe thalassemia in 1925, identifying a unique form of severe anemia among Italian children who had also developed characteristic bone malformations and splenomegaly (Cooley and Lee, 1925). The characterization of Cooley's anemia continued throughout the Mediterranean community where it was believed to be endemic, and the disease was named thalassemia, from thalassa, the Greek word for sea (Cooley et al., 1927; Koch and Shapiro, 1932; Whipple and Bradford, 1932; Bradford and Dye, 1936; Whipple and Bradford, 1936; Ellis et al., 1954). Eventually, it was discovered that thalassemia is a genetic disorder, not limited to Mediterranean Europe, but actually found worldwide in a variety of forms. In fact, thalassemia has been identified as the most common monogenic disease in humans (Weatherall and Clegg, 1996).

Thalassemia has been divided into four clinical syndromes. The silent carrier state includes people who carry a thalassemia mutation on only 1 thalassemia gene, and who are completely asymptomatic. Others carry a thalassemia mutation on only one gene, but are not entirely asymptomatic and develop minor thalassemia symptoms, defining the thalassemia trait syndrome. Homozygotes or compound heterozygotes for thalassemia are divided among the remaining two syndromes, based on the severity of disease. Thalassemia major is defined by early onset of severe disease requiring regular blood transfusions to survive. Those who do not require transfusions, with delayed presentation of symptoms, characterize the Thalassemia intermedia syndrome.

1.4.1 Ineffective, Hyperactive Erythropoiesis and Clinical Manifestation of Thal

The genetic disorder in thalassemia causes a decrease or complete lack of production of beta-globin or alpha-globin protein molecules if the genetic abnormality is within the β-globin gene (on chromosome 11) or α-globin gene (on chromosome 16), respectively, or globin gene regulatory region. In humans, two β-globin molecules normally assemble with two α-globin molecules to form normal adult hemoglobin A, the predominant hemoglobin beyond the age of approximately 6 months (Figure 1.14). In thalassemics, an imbalance in globin production disrupts red cell production, resulting in
the uncontrolled augmented production of defective red blood cells, and release of immature red cells from the erythroid marrow. In severe forms of thalassemia, as much as a 10-fold increase in erythropoiesis is achieved when untreated, of which 95% of the cells produced may be ineffective (Nathan and Gunn, 1966; Olivieri, 1999). Deleterious effects of the resulting hyperplastic erythroid marrow include the increased absorption of iron, to supply the increased demand of iron due to the augmented production of red blood cells, which accumulates in tissue stores beyond toxic thresholds. Furthermore, the physical pressure of the hyperactive marrow, expanding to as much as 30 times its normal volume within medullary cavities, causes severe skeletal abnormalities, including the characteristic thalassemia bone malformations of the skull and face (Weatherall and Clegg, 1981; Cazzola and Finch, 1989; Olivieri, 1999). Furthermore, iron overload becomes an even greater concern for patients with severe thalassemia syndromes receiving life-sustaining regular transfusions, who store and rapidly accumulate iron originally incorporated within the hemoglobin of transfused red blood cells.

![Diagram of hemoglobin synthesis](image)

**Figure 1.14.** (a) The α- and β-globin gene clusters on chromosomes 11 and 16, and the various hemoglobin molecular combinations (Kulozik, 1999) (b) Normal pre- and post-natal globin-chain production in humans, illustrating the progressive switch from fetal to adult hemoglobin production throughout the first 8 months after birth (Olivieri, 1999).
1.4.2 Regular Transfusion Therapy

In the 1960s, a major clinical breakthroughs in the treatment of thalassemia major was the realization that regular transfusions, aimed at maintaining elevated hemoglobin levels, significantly improved the clinical course in patients with thalassemia, compared to those receiving irregular (crisis-dependant) transfusions (Orsini and Boyer, 1961; Wolman, 1964; Wolman, 1969)

1.4.2.1 Indications

Due to the phenotypic variation observed in thalassemia, genotype is not a reliable indicator of clinical severity, and the initiation of transfusion is usually based on a hemoglobin concentration consistently below 6 g/dl for three months, combined with poor growth, splenomegaly, and marrow expansion (Olivieri and Weatherall, 1999). Children with severe forms of thalassemia characteristically develop complications associated with anemia, ineffective erythropoiesis, and splenomegaly, within the first year of life. Regular red cell transfusions correct and manage the original symptoms of thalassemia, permitting normal growth and development throughout childhood (Piomelli et al., 1969; Piomelli et al., 1995; Cazzola, 1995; Cazzola, 1997; Olivieri and Brittenham, 1997; Rund, 2000).

1.4.2.2 Strategy and Transfusion Regimen

The strategy of regular red blood cell transfusion therapy is to prevent hypoxia, and suppress endogenous erythropoiesis. Without transfusion therapy, children with severe thalassemia syndromes develop life-threatening complications of anemia and ineffective erythropoiesis (Weatherall and Clegg, 1981). The early initiation of a regular transfusion regimen designed to maintain baseline (pretransfusion) hemoglobin around 9.5g/dl, could effectively (1) reverse and prevent hypoxia (protecting against cardiomegaly), and (2) suppress endogenous erythropoiesis, preventing bone
malformations due to bone marrow expansion, and preventing or at least delaying hypersplenism (hyperactive spleen struggling to filter defective red blood cells and reticulocytes) in patients with Thalassemia major (Piomelli et al., 1969; Piomelli et al., 1995; Cazzola 1995; Cazzola 1997; Rund 2000). Alternative supertransfusion regimens designed to maintain baseline hemoglobin’s above 11 g/dl have been proposed, but have not proven to be any more effective, while increasing transfusional iron accumulation (Cazzola et al., 1995). Neocyte (young red cell) transfusions have been proposed to extend the interval between transfusions and minimize transfusional iron accumulation. However, in clinical practice, neocytes have only been able to extend the transfusion interval by only 13-16%, while costing 3 times as much (per unit) as normal packed red cells (Cohen et al., 1983; Marcus et al., 1985).

Transfusion of 15 mL of blood / kg body weight (mL/kg), using transfused blood with a hematocrit of 0.7, is equivalent to the transfusion of 10.5 mL of red blood cells / kilogram body weight. At this concentration, the total volume of red cells transfused contains enough hemoglobin to raise the patients’ hemoglobin concentration by approximately 4g/dl. Regular transfusion at 3 to 4 week intervals, based on a life span of transfused red cells of approximately 12 weeks, would theoretically maintain a pre-transfusion (baseline) hemoglobin of approximately 9.5 g/dl.

1.4.3 Iron Accumulation and Overload

In thalassemia, the imbalance in globin chain synthesis is responsible for stimulating increased erythropoiesis in the bone marrow, which is associated with an increase in iron absorption, as the body anticipates the production of hemoglobin molecules. Therefore, even in untransfused thalassemia patients, iron overload gradually occurs. In the severe forms of thalassemia requiring life-preserving regular red cell transfusion therapy, endogenous erythropoiesis and iron absorption is suppressed by maintaining baseline (pretransfusion) hemoglobin levels above 9.5 g/dl (Cazzola 1995). However, iron overload develops very rapidly, as each milliliter of transfused red cells carries 1 milligram of iron into the body (Reed and Vichinsky, 1999).
In 1936, one of the earliest papers characterizing thalassemia reported increased body iron levels in irregularly transfused children with thalassemia who died of progressive anemia when they were between 5 to 7 years of age (Whipple and Bradford, 1936). Hepatic iron concentrations measured at autopsy ranged between 3.8 to 13.2 mg/g, dry weight, reflecting the combined loading of transfusional and absorbed iron. Although occasional blood transfusions appeared to improve the health and extend the survival of patients with thalassemia, Ellis et al. (1954) reported the accumulation of iron in the organs of patients with thalassemia who had received various amounts of transfused blood, and also identified associated liver fibrosis. In 5 patients who died between 4 and 27 years of age, and 7 patients biopsied between 3 and 15 years of age, extensive hepatic siderosis was identified histologically. Liver fibrosis was also identified in 11 of these 12 cases. Hepatic iron concentrations were available in only 2 cases, and were both elevated at 7.6 and 8.1 mg/gww (equivalent to 25.3 and 27.0 mg/g,dw; respectively, assuming 1gww = 0.3g,dw, assuming the liver is approximately 70% water (Brittenham et al)). Witzleben and Wyatt (1961) also reported 4 out of 5 deaths, within 13 years of life in patients with β-thalassemia major who received repeated transfusions. All developed cirrhosis, including the one patient that was still alive at 16 years of age. Final hepatic iron concentrations from cirrhotic livers were reportedly between 27.7 and 81.4 mg/g,dw (cirrhosis appears to affect the reliability of HICs in reflecting the body iron level). These three papers were the earliest studies revealing the development of iron overload and early death in patients with thalassemia, and identifying liver damage associated with iron overload.

Over the last 40 years, while struggling to find the optimal regular transfusion therapy regimen, Engle (1964) reported that thalassemia patients receiving transfusions acquired cardiac disease at a mean of 16 years of age, and that 50% of those diagnosed with cardiotoxicity died within 3 months. Ehlers et al (1980) also reported on cardiac disease in a group of patients at a mean age of 18 years, and death within 3 months of detecting cardiotoxicity, despite advances in transfusion and cardiac therapy. Wolfe et al (1985) also identified cardiac disease at a mean age of 19.2 years in regularly transfused thalassemia patients who were not compliant with regular DFO chelation therapy, while those who were compliant with chelation for six years, were enjoying cardiac disease-
free survival as they approached their nineteenth birthday. Therefore, it has been well-established that regularly-transfused patients with thalassemia who are inadequately chelated, will rapidly accumulate iron in their bodies, and suffer various complications of iron overload (discussed in Section 1.1.7), eventually developing life-threatening cardiac disease in the second or third decade of life.

1.4.4 Iron Chelation Therapy in Thalassemia

A tremendous breakthrough in 1964, was the development of the iron chelator deferoxamine, to manage iron accumulation and overload in regularly transfused patients (Keberle, 1964). In the late 1970s, deferoxamine became the standard therapy, wherever available, for managing and preventing transfusional iron overload (See Section 1.3.2).

Several studies over the past 20 years have focussed on the impact of deferoxamine chelation therapy in effectively reducing morbidity and mortality in regularly-transfused patients with thalassemia. Studies have revealed that chelation therapy can prevent and correct both liver disease and heart disease, extending survival in regularly-transfused patients with thalassemia, while minimizing the risk of developing other iron-related endocrinopathies (Flynn et al., 1982; Modell et al., 1982; Weatherall et al., 1983; Pippard and Callender, 1983; Freeman et al., 1983; Marcus et al., 1984; Zurlo et al., 1989). Brittenham et al. (1994) suggested a decreased risk of developing diabetes mellitus in thalassemia patients introduced to adequate chelation therapy at an early age. Bronspeigel-Weintrob et al. (1990) first reported that DFO therapy appeared to prevent growth failure and gonadal dysfunction. Jensen et al. (1995) also observed an increase in fertility among men and women with thalassemia receiving chelation therapy. However, other studies have identified several chelated thalassemia patients with gonadal dysfunction, and females experiencing secondary amenorrhea despite previous evidence of normal pituitary function (Olivieri and Brittenham, 1997).

Two recent long-term studies in patients with thalassemia have confirmed that with adequate DFO chelation therapy, normal disease-free survival can be achieved (Brittenham et al., 1994; Olivieri et al., 1994).
1.4.5 Serum Ferritin Concentration and Thalassemia

The most common method of assessing and monitoring body iron levels in patients with thalassemia, has been using serum ferritin concentration. However, there is a growing awareness that serum ferritin concentration may not be an accurate and reliable indicator of body iron burden. In thalassemia, reports have revealed a correlation between serum ferritin concentration and hepatic iron concentration (through SQUID or liver biopsy) ranging from as low as $r=0.16$ to as high as $r=0.96$ (De Virgillis et al., 1980; Aldouri et al., 1987). In children with thalassemia, De Virgillis et al. (1980) reported no correlation between serum ferritin concentration and both the numbers of transfusions ($r=0.176$, p-value reported as not significant) and the hepatic iron concentration ($r=0.159$, p-value reported as not significant). Worwood et al (1980) reported a correlation between SFC and the volume of blood transfused of only $r=0.38$ among regularly transfused thalassemia major patients. Other, more recent, studies have reported serum ferritin correlation with hepatic iron concentration ranging between 0.55 to 0.76 in patients with $\beta$-thalassemia (Brittenham et al., 1989; Brittenham et al., 1993; Nielsen et al., 1995; Piga et al., 1999). Although statistically significant, the amount of variability observed in these studies suggests serious limits on the clinical reliability of measures of SFC. However, stronger correlations as high as $r=0.96$ have also been reported in patients with thalassemia (Prieto et al., 1975; Aldouri et al., 1987). Factors that influence SFC that are common in thalassemia patients include inflammation, infection, ascorbate deficiency, and liver disease (Gordeuk et al., 1987).
1.5 Pathophysiology of Sickle Cell Disease

Sickle cell disease is a group of genetic diseases characterized by the production of abnormal sickle hemoglobin S (HbS). Normally, beyond the age of 6 months, more than 95% of circulating hemoglobin is “adult hemoglobin” (HbA), a tetrameric polypeptide complex comprised of 2 α-globin and 2 β-globin molecules. In sickle cell disease, a specific point mutation within the β-globin gene on chromosome 11 results in the translation of abnormal βS-globin molecules, which combine with normal α-globin molecules to form sickle hemoglobin (HbS) instead of normal adult hemoglobin (HbA) (Pauling, 1949). Specifically, a single base mutation (A→T) within the DNA nucleotide triplet coding for the sixth amino acid from the N-terminus of the β-globin protein, inserts valine instead of glutamic acid during B-globin translation.

The most common genotypes of sickle cell disease, all manifesting the production of HbS at varying levels, include (1) homozygous SS sickle cell disease, (2) compound heterozygous sickle cell-hemoglobin C (SC) disease, and (3) compound heterozygous sickle cell-β-thalassemia. Less common genotypes are the compound heterozygous Hb SD and Hb SOArab. In homozygous SS sickle cell disease the βS-globin (S) gene is inherited from both parents, and the genotype is associated with greater morbidity and mortality. In SC disease, the βS-globin (S) gene is inherited from one parent, and the βC-globin (C) gene is inherited from the other. SC disease is associated with a relatively milder phenotype and is frequently undiagnosed (Serjeant, 1999). Sickle cell-β-thalassemia results from the inheritance of a βS-globin (S) gene from one parent, and a β-thalassemia gene from the other. If a more severe β°-thalassemia gene is inherited, incapable of producing any functional β-globin chains, sickle cell-β°-thalassemia is associated with a severe phenotype similar to SS disease. However, if a milder β°-thalassemia gene is inherited, capable of synthesizing some functional β-globin chains, a milder phenotype is displayed as a condition similar to SC disease.

Sickle cell trait is a condition defined by the inheritance of a βS-globin gene from one parent, and a normal β-globin gene from the other. In this condition, sickle hemoglobin is produced, however concentrations of HbS remain between 20-45% of total
hemoglobin (Serjeant, 1999). At these concentrations, polymerization of HbS molecules has not been observed under normal oxygenation, and the condition is believed to be harmless.

1.5.1 Abnormal Erythrocytes and Hemoglobin S

The erythrocytes of patients with sickle cell disease contain high concentrations of HbS molecules, which aggregate and form long, rigid polymers under deoxygenated conditions (Figure 1.15). As a result, the internal viscosity of the red cell increases, provoking changes in the cell membrane, and the rigid polymers deform the cell into its characteristic sickle shape. Sickled cells are either destroyed prematurely, with a mean red cell survival of approximately 10 to 15 days (compared to normal mean red cell survival of 120 days) which causes anemia (hemoglobin levels usually ranging between 6 – 9 g/dl), or may get trapped within capillaries, restricting blood flow and causing tissue hypoxia. This vaso-occlusion, at various sites in the body, causes the severe symptoms of sickle cell disease.

At high intracellular concentrations, the HbS molecule behaves as a low-affinity transporter of oxygen. The molecule becomes highly saturated in the lungs, and is capable of delivering more oxygen to peripheral tissues than HbA.

Figure 1.15. Polymerization of sickle hemoglobin (HbS) in deoxygenated environments causing sickling of red blood cells and vaso-occlusion (Steinberg, 1999).
1.5.2 Clinical Manifestations

The double-edged sword of sickle cell disease is the rapid destruction of abnormal red cells (lowering hemoglobin levels and causing anemia) combined with the vaso-occlusive tendencies of circulating sickled cells.

Clinical complications related to rapid red cell destruction include acute and chronic anemia, gallstone formation, fever and infection. The spleen is the principle organ involved with the removal of defective red cells and the filtration of intravascular bacteria. In sickle cell disease, splenic function is exhausted through its efforts in coping with the excess of defective red cells, and bacterial filtration is compromised. A common cause of death among sickle cell patients is an acute splenic sequestration crises, associated with splenic enlargement and a rapid hemoglobin drop of between 2 – 6 g/dl, as red cells get trapped in the spleen (Serjeant, 1999). Chronic hypersplenism is another common complication in sickle cell disease, characterized by sustained splenomegaly and bone marrow hyperactivity, baseline hemoglobin levels between 3 – 5 g/dl, reduced platelets, and red cell survival of 2 – 3 days. The greatest effect may be the increased metabolic demands of the hyperactive marrow, which may compromise growth. Bacterial filtration is also compromised in the spleen as normal splenic function is lost. Sickle cell patients are much more prone to blood-borne bacterial infection, especially pneumococci. Also common among sickle cell patients under 15 years of age, is an episodic aplastic crisis related to parvovirus infection, which suppresses bone marrow activity. Furthermore, chronic hemolysis in sickle cell disease is responsible for the development of pigment gallstones, which may cause acute or chronic cholecystitis, or obstruct the cystic duct or common bile duct.

Clinical complications related to vaso-occlusion include avascular necrosis of the bone marrow, causing painful episodes of swelling in the bones of the hands and feet (dactylitis), long bones, spine, pelvis, and femoral head. These episodes begin as early as 3 months of age and frequently are recurrent. Vaso-occlusion also commonly affects the skin (causing ulcers, often on the legs), the corpura cavernosa (causing priapism), the retina, and the brain (causing stroke). Another major cause of morbidity and mortality in
sickle cell disease, and the largest contributor to death after the age of 2 years, is the combination of pulmonary sequestration, infarction, embolism, and infection, known as the acute chest syndrome.

1.5.3 Regular Transfusion Therapy

Regular red blood cell transfusion therapy improves survival and reduces morbidity in patients with sickle cell disease (Powars et al., 1978; Emond et al., 1985; Wang et al., 1991; Styles et al., 1994; Pegelow, 1995; Olivieri, 1997; Adams, 1998; Vichinsky et al., 1998; Weatherall, 2000; Ohene-Frempong, 2001).

1.5.3.1 Indications

The indications for regular red cell transfusion therapy in patients with sickle cell disease are expanding, the most common being an increased risk of stroke (i.e. history of stroke or repeated abnormal internal carotid and middle cerebral artery blood flow velocity detected by transcranial doppler ultrasonography) (Powars et al., 1978; Adams et al., 1998), lung disease (i.e. recurring acute chest syndrome) (Vichinsky et al., 1998), frequent painful vaso-occlusive crises, and recurrent acute splenic sequestration (Ohene-Frempong, 2001; Emond et al., 1985). Powars et al. (1978) reported that between 50 – 70% of sickle cell patients who had a stroke, experienced a recurrent stroke within 3 years.

1.5.3.2 Strategy and Transfusion Regimen

The prophylactic strategy involves replacing the production and circulation of endogenous red blood cells containing sickle hemoglobin (HbS), with healthy transfused blood cells containing normal adult hemoglobin (HbA). Reducing and maintaining the level of Hb S at less than 30% of total circulating hemoglobin minimizes the number of sickled, rigid, vaso-occlusive red cells responsible for sickle cells crises. Although never tested by clinical trial, studies have concluded that maintaining the level of sickle
hemoglobin at less than 30%, through prophylactic transfusion, effectively protects sickle cell patients at high risk of stroke (Wang et al., 1991; Pegelow et al., 1995; Cohen et al., 1992).

1.5.4 Iron Overload and Associated Complications

The major caveat of red cell transfusion therapy is the accumulation of iron in the body, due to the lack of appropriate physiological mechanisms of eliminating iron. Disease complications and early death have been associated with increased iron overload in patients with B-thalassemia (Brittenham et al., 1994; Olivieri, 1994). This has raised the concern about the effects of iron overload in patients with sickle cell disease, and the rate of transfusional iron accumulation.

Direct and indirect methods of assessing body iron burden are available (see section 1.2). Serum ferritin concentration continues to be the most commonly used indirect estimator of body iron overload (Brittenham, 1993; Finch, 1994; Olivieri and Brittenham, 1997). However, evidence has accumulated against the reliability of the serum ferritin concentration in sickle cell disease (Vieira et al., 1999; Harmatz et al., 2000). Recurrent fever, acute infection, and chronic inflammation, all common in patients with sickle cell disease, also influence the serum ferritin concentration, complicating its interpretation (Olivieri and Brittenham, 1997). Direct measures of hepatic iron concentration, through biochemical assay or SQUID magnetic susceptometry, provide the most reliable quantitative assessment of body iron burden (Pippard, 1989, Angelucci, 2000). Interpretation of hepatic iron concentrations is thoroughly described in section 1.2.2.

Earliest reports of iron overload in patients with sickle cell disease receiving multiple blood transfusions include Frumin and Miller (1952), who identified a striking amount of iron deposits within the liver of a four-year old sickle cell patient who had received 18 liters of transfused blood over 3 years. Hepatic dysfunction, fibrosis, and cirrhosis associated with the deposition of iron contained within transfused blood was also identified in patients with sickle cell disease (Green et al. 1953; Bogoch et al. 1955; Finch et al., 1982). Cardiac iron deposits were also identified in a heavily transfused patient with sickle cell disease (Buja and Roberts, 1971), suggesting that in sickle cell
disease, transfusional iron overload causes cardiac dysfunction, failure, and death. Transfusional iron overload has also been implicated as the source of massive iron deposits in endocrine organs, and cause of endocrine disease in sickle cell disease (Buja and Roberts, 1971; Finch et al., 1982). Several studies have confirmed the progression of transfusional iron overload in sickle cell disease. However, these studies could not accurately quantify the rate of iron accumulation, either because they used indirect and unreliable indicators of body iron levels, or due to the fact that several patients were receiving chelation therapy (Washington and Boggs, 1975; Hussain et al., 1978; Buffone et al., 1980; Rao et al., 1984; Olivieri et al., 1997; Harmatz et al., 2000).

Recent reports are suggesting that elevated body iron levels associated with transfusion therapy, may be less toxic in patients with sickle cell disease in comparison to thalassemia (Harmatz et al., 2000; Harmatz et al., 2000). However, iron toxicity is unlikely to be exclusively dependent upon the concentration or amount of iron in the body. Other factors probably include:

- the rate of iron accumulation
- the distribution of iron within the various storage compartments
  (ie. reticulo-endothelial and parenchymal)
- how long patients are exposed to elevated iron levels
- age at initial exposure to elevated iron levels
- viral hepatitis
- ascorbate status
- alcohol consumption

Furthermore, the rate of iron accumulation in regularly transfused patients with sickle cell disease has never been quantitatively reported, and guidelines for the initiation of chelation therapy are lacking.
1.5.5 Iron Chelation Therapy in Sickle Cell Disease

Although very few studies have focused on the impact of iron chelation therapy on patients with sickle cell disease, the results have suggested that the outcome will be similar to that observed in regularly transfused and chelated patients with thalassemia. Increased urinary iron excretion using intramuscular and intravenous DFO therapy, and net negative iron balance using 12-hour subcutaneous and 18-hour intravenous infusion in sickle cell disease has been reported (Cohen and Schwartz, 1978; Cohen and Schwartz, 1979; Cohen et al., 1989; Sillman et al., 1993). Increased urinary iron excretion was also identified in regularly transfused sickle cell patients chelated with deferiprone (L1), and paralleled urinary iron excretion using DFO. However, the additional fecal iron excretion observed using DFO, suggests that it is a more effective and efficient iron chelator (Collins et al., 1994). Therefore, iron chelation therapy introduced to patients with sickle cell disease receiving regular transfusions should be able to stop the accumulation of body iron, reduce the amount of iron in the body, and prevent iron-induced organ damage. However, due to the toxic effects of premature chelation therapy (when body iron levels are low), the challenge remains in deciding when to begin chelation therapy, as the rate of iron accumulation is not clear.
Chapter 2

RATIONALE, OBJECTIVES, HYPOTHESES

2.1 Rationale

For the last forty years, clinicians caring for patients with thalassemia and/or sickle cell disease, have been struggling with the progression of iron overload in response to regular transfusion therapy. Clearly, after prolonged exposure to toxic iron levels, patients begin to develop myriad complications related to iron-overload, most notably liver disease, endocrinopathies, cardiac disease, and early death. For the last 20 years subcutaneous DFO therapy has been the standard treatment of transfusional iron overload with proven efficacy. However, due to its toxic effects when body iron levels are not seriously elevated, chelation therapy cannot be simply initiated together with transfusion therapy. Similarly, DFO therapy must be suspended when body iron levels have been appropriately reduced. Therefore, clinicians try to monitor body iron levels and introduce chelation therapy when appropriately elevated body iron levels are established. The problems are:

1. Clinicians have been unable to appropriately anticipate the progression of iron overload, because the rate of iron accumulation in regularly transfused patients with these disorders has never been appropriately and quantitatively reported.

2. Clinicians are continuing to use indirect and unreliable indicators of body iron burden, specifically the serum ferritin concentration, because direct and accurate techniques are believed to be invasive (requiring liver biopsy) or have been poorly available (SQUIDs).

3. Since the efficiency with which DFO actually removes iron from the body is not known, quantitative guidelines for prescribing chelation therapy and achieving iron balance are lacking.
Furthermore, it has recently been suggested that patients with sickle cell disease may not be as susceptible to the complications of iron overload as patients with thalassemia, through a "protective" mechanism possibly related to iron distribution among parenchymal and reticulo-endothelial cell stores. Without more conclusive evidence, it remains unclear if less intensive chelation therapy would be appropriate for patients with sickle cell disease.

Improving the management of these patients relies on identifying highly accurate and appropriate methods of monitoring body iron levels, and improving guidelines and strategies for iron chelation therapy.

2.2 Objectives

The purposes of this study were:

1a) To reveal the safety of ultrasound-guided liver biopsies.
1b) To reveal the success of our liver biopsy program.

2a) To quantify the initial rate of iron accumulation in patients with sickle cell disease and thalassemia introduced to regular red blood cell transfusion therapy, and identify how quickly iron levels accumulate beyond toxic thresholds.
2b) To identify similarities and differences in iron accumulation and distribution between sickle cell disease and thalassemia.
2c) To identify body iron concentrations associated with liver damage in both sickle cell disease and thalassemia.
2d) To reveal the reliability of serum ferritin as an indicator of body iron levels.

3a) To calculate the chelation efficiency of DFO and L1.
3b) To define clinically-useful algorithms to calculate doses of chelation required to achieve iron balance.
The overall objective is to identify the importance and value of hepatic iron quantitation, which is no longer just the most accurate indicator of body iron levels, but can now also converted to a reliable quantitative measure of the total amount of iron stored in the body. Hepatic iron concentration clearly identifies patients who should and should not be chelated, helping clinicians decide when to initiate chelation therapy. Also, hepatic iron concentrations can now be used to calculate required doses of chelation to achieve iron balance. If we can prove that liver biopsies are safe, and a successful biopsy system can be realized. clinicians may be persuaded to use hepatic iron concentrations.

2.3 Hypotheses

1. The safety and reason for incorporating liver biopsies into a hemoglobinopathy program. We hypothesized that US-guided liver biopsy is a safe and minimally-invasive procedure, as determined by i) a near negligible rate of morbidity and no mortality, and ii) a well-established and well-accepted system of periodic liver biopsy. We also hypothesized that US-guidance improves the success of the procedure in obtaining adequate samples of tissue for both iron quantitation and histological assessment.

2. The rate of transfusional iron accumulation (pre-chelation).
Since patients with sickle cell disease and thalassemia introduced to transfusion therapy were receiving similar amounts of transfusional iron (per kilogram body weight), we hypothesized that they would experience similar rates of iron accumulation. We also hypothesized that patients could reach toxic iron concentrations within 12 months of starting regular transfusions.

3. Similarities of iron accumulation in sickle cell disease and thalassemia.
We hypothesized that in both groups, similar patterns of iron distribution and liver damage would be observed, since both groups are i) being introduced to transfusion therapy, ii) receiving similar amounts of transfusional iron, iii) receiving transfusions at the same periodic interval. There is no evidence suggesting a different mechanism of iron accumulation in either disorder.
4. The reliability of the serum ferritin concentration.

We hypothesized that serum ferritin concentration would be a poor indicator of iron overload in both sickle cell disease and thalassemia, mainly due to various other physiological influences common among hemoglobinopathy patients including ascorbate deficiency, fever, infection, inflammation, liver damage, and ineffective erythropoiesis.

5. The chelation efficiency of deferoxamine and deferiprone.

Based on a recently reported near-perfect correlation between the amount of iron contained with the total body iron store and the hepatic iron concentration (Angelucci et al., 2000), we hypothesized that we could calculate the chelation efficiency of DFO and L1 based on serial HICs, and transfusion and chelation records. Since iron balance studies have suggested that L1 is a less efficient iron chelator than DFO, we hypothesized that DFO would have greater chelation efficiency, and L1 chelation efficiency would show how the chelator is unable to achieve iron balance at clinically-prescribed doses. Chelation efficiency can be factored into equations for predicting iron balance, and defining appropriate chelation doses and regimens.
Chapter 3

ULTRASOUND-GUIDED PERCUTANEOUS LIVER BIOPSIES
IN HEMOGLOBINOPATHY PATIENTS:
A TEN-YEAR EXPERIENCE IN CHILDREN AND ADULTS

3.1 Background and Literature Review

Hemoglobinopathy patients rapidly accumulate iron into their bodies once they begin regular red blood cell transfusion therapy. Due to poor physiological mechanisms of iron excretion, these patients are introduced to life-preserving chelation therapy to help them eliminate excess iron. Closely and accurately monitoring body iron levels is crucial to disease-free survival in these patients. Section 1.2 summarizes several of the direct and indirect techniques used to assess and monitor body iron levels in these patients.

Biochemical iron quantitation of hepatic tissue obtained through biopsy remains the gold standard technique for accurately evaluating body iron levels. The only other direct technique that is as reliable as biochemical quantitation is magnetic susceptibility of the liver, using a Superconducting Quantum Interference Device (SQUID) (Brittenham et al., 1982; Brittenham, 1988; Piga et al., 1999). However, the lack of availability of SQUIDs globally, due to its complex design and function, has limited its clinical use.

Although clinicians realize that hepatic iron quantitation is the most accurate and reliable method of monitoring body iron levels in their hemoglobinopathy patients, what remains unresolved is whether the invasiveness of liver biopsies make them inappropriate for periodically monitoring body iron levels. Many clinicians have felt that indirect imaging techniques and serological markers of iron overload (ie. serum ferritin and transferrin concentrations) are reliable, and therefore the invasiveness and risks associated with a liver biopsy cannot be justified. However, clinicians are beginning to realize that these surrogate markers are actually unreliable, due to various physiological influences (ie. infection, inflammation, ascorbate deficiency, liver damage), which are common in hemoglobinopathy patients (Roeser et al., 1980; Baynes et al., 1986; Brittenham et al, 2001).
The first liver biopsy on record was performed by Paul Ehrlich in 1883 (Ehrlich, 1884), and the first series was published in 1907 (Schupfer, 1907), reporting the successful acquisition of liver tissue from percutaneous biopsy. In 1958, Menghini reported a "one second" liver biopsy technique, after identifying a relationship between the duration of the intrahepatic phase of the biopsy and the rate and severity of complications (Menghini, 1958). With growing acceptance of Menghini's liver biopsy technique, Frank and Leodolter (1966) were the first to report that percutaneous liver biopsy could safely be performed as an out-patient procedure. Several clinical trials have since reported on morbidity and mortality associated with percutaneous liver biopsy (Table 1.2), investigating the safety of out-patient biopsy and the impact of ultrasound (pre-procedure or guidance) on safety and ability to acquire adequate tissue. Two recent review papers also describe alternative liver biopsy techniques, indications, contraindications and recommendations (Grant and Neuberger, 1999; Bravo et al., 2001).

This comprehensive review of the literature reveals a liver biopsy mortality rate ranging from 0.01% to 0.4%, and a rate of significant morbidity ranging from 0.2% to 3.5%.

The purpose of this study is to reveal the success of our liver biopsy program over the last 10 years (1990-2000) performing liver biopsies on haemoglobinopathy patients. A successful liver biopsy in our program is defined by the combination of 4 factors:

1. The operator or interventionalist obtaining an adequate amount of tissue.
2. The tissue being appropriately divided into an iron and a histology sample.
3. Proper processing and a successful reliable biochemical assay.
4. A definitive diagnosis reported by our pathologists.

Another aim of this study was to analyze the relative cost-effectiveness of using ultrasound-guided liver biopsies for monitoring body iron levels, and compare the average cost of these biopsies to the cost of using monthly serum ferritin concentrations. We hope to convince clinicians that the invasiveness of liver biopsies is minimized when performed percutaneously using ultrasound-guidance, and that this procedure is capable of safely obtaining tissue for iron quantitation and also obtaining tissue for histological assessment.
3.2 Experimental Design

The medical records of all patients attending the pediatric and adult haemoglobinopathy programs between May 1990 and December 2000 were reviewed. We identified the date of each biopsy, tissue sample weights, pathology reports, and any comments of biopsy-related complications. Percutaneous liver biopsy data, including dates, technique, equipment, and outcome (complications), was also prospectively recorded since 1992 by our pediatric interventional radiologists.

3.2.1 Sample Population

A total of 296 patients had 911 percutaneous liver biopsies at a mean (±SEM) age of 18.59 ± 0.37 years (median: 16.55, range: 0.84 – 73.17 years). Table 3.1 reveals the underlying diagnoses in these 296 patients.

Table 3.1. Diagnoses of 296 Hemoglobinopathy Patients Who Had Percutaneous Liver Biopsies

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Thalassemia major</td>
<td>157</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>46</td>
</tr>
<tr>
<td>Diamond Blackfan anemia</td>
<td>22</td>
</tr>
<tr>
<td>Thalassemia intermedia</td>
<td>19</td>
</tr>
<tr>
<td>E-Thalassemia</td>
<td>13</td>
</tr>
<tr>
<td>Pyruvate Kinase Deficiency</td>
<td>5</td>
</tr>
<tr>
<td>Hemoglobin-H disease</td>
<td>6</td>
</tr>
<tr>
<td>α-Thalassemia</td>
<td>4</td>
</tr>
<tr>
<td>Other chronic anemia</td>
<td>24</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>296</td>
</tr>
</tbody>
</table>

3.2.2 Ultrasound-Guided Percutaneous Liver Biopsy

All biopsies were performed as out-patient procedures, except when combined with other more invasive therapeutic procedures (ie splenectomy, central line insertion).
Since 1993 at our pediatric center and since 1998 at our adult center, all out-patient biopsies have been performed using ultrasound guidance.

All percutaneous liver biopsies are scheduled as outpatient procedures, and are routinely performed in the morning. For patients who are regularly transfused, the biopsy is scheduled on the same day as a transfusion. Shortly after patients arrive at the hospital, blood samples are obtained to identify any potential risk of bleeding, including a check of prothrombin time, partial thromboplastin time, platelet count, international normalized ratio, and hemoglobin levels. Written informed consent for the procedure is obtained from the patient, a parent or guardian. Patients are administered a combination of sedatives, using intravenous meperidine hydrochloride (Demerol; Abbott Laboratories, Saint-Laurent, Quebec) and either intravenous pentobarbital sodium (Nembutal sodium; Abbott Laboratories) or intravenous diazepam (Diazemuls; Pharmacia and Upjohn, Mississauga, Ontario). Patients are then transported to the procedure room, and an abdominal ultrasound is performed to visualize the underlying anatomy and define the biopsy site. Using sterile techniques, EMLA cream is applied around the chosen puncture site, then a local anaesthetic is also injected subcutaneously. Using a subcostal midline approach with ultrasound guidance, the biopsy needle is directed into the liver, carefully avoiding the surrounding anatomy and vasculature, and a core of tissue is obtained. Between 2-3 needle passes are usually performed to obtain sufficient tissue. The types of biopsy needles used are listed in Table 3.2. Pressure is applied at the biopsy sites for 2-3 minutes, then a bandage placed over the puncture wound. Post-procedure, patients are transferred for recovery and monitoring for 4 - 6 hours, and regularly transfused patients also receive their scheduled transfusions.

<table>
<thead>
<tr>
<th>Needle Type</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>18G Angiomed Autovac†</td>
<td>Bard, Mississauga, Ontario</td>
</tr>
<tr>
<td>18G Surecut‡</td>
<td>TSK Laboratories, Tokyo, Japan</td>
</tr>
<tr>
<td>18G Biopty*</td>
<td>Bard, Mississauga, Ontario</td>
</tr>
</tbody>
</table>

†automated, end-cutting, variable 2-, 3-, or 4-cm core needle
‡non-automated, end-cutting core needle
*automated, side-cutting, 2.2-cm core needle
3.2.3 Specimen Handling

Liver tissue is divided and sent to two different laboratories. One tissue sample is immediately placed in formalin and sent for local histological assessment. The remainder of the tissue is lightly rinsed with saline, then placed into a dry iron-free Eppendorf tube and immediately transferred to a -70°C freezer. Frozen samples are periodically batched and shipped to an external laboratory for iron quantitation.

3.2.4 Adequacy of Samples for Iron Quantitation and Histological Assessment

The weight of tissue samples used for iron analysis were obtained for 873 liver biopsies, and samples were considered adequate for iron quantitation if the dry weight of the tissue was ≥1.0mg (Angelucci, 2000). Pathology reports were also obtained for 894 liver biopsies. Tissue samples for pathology were considered adequate if a definitive histological description of hepatic fibrosis, cirrhosis, and portal inflammation was reported. For iron and pathology samples, the ability to obtain adequate tissue using ultrasound guidance was compared to the blind biopsy technique.

3.2.5 Cost Analysis

The relative cost of using ultrasound-guided liver biopsies for monitoring body iron levels was compared to the average to that of monthly serum ferritin concentrations. The mean interval between serial biopsies observed in this series, defined the number of months of serum ferritin measures used in this cost comparison. Direct and indirect patient costs were obtained through the Interventional Radiology department and the Purchasing department at the Hospital for Sick Children, and through the Ministry of Health.
3.3 **Statistical Analysis**

Descriptive statistics including the mean, standard error of the mean, median, and range, were calculated for the age at biopsy, number of biopsies, the duration of the interval between biopsies, and the biopsy sample dry weights.

### 3.3.1 Group Comparisons (Two Sample t-test and Fisher Exact test)

The two-sample t-test (without the assumption of equality of variances) was used to reveal significant differences between groups when comparing means and distributions (Dixon, 1985; Pagano, 2000). Parameters reporting frequencies or ratios were compared using the Fisher exact test. All tests were two-tailed, using a significance level of 0.05.

For iron and pathology samples, the success at obtaining adequate tissue before the use of ultrasound-guidance was compared to the success at obtaining adequate tissue after ultrasound-guidance became standard technique, in an effort to compare the ultrasound-guided technique to the blind biopsy technique.

### 3.4 Results

911 liver biopsies were performed on 296 hemoglobinopathy patients at a mean age of 18.59 ± 0.37 years (median: 16.55; range: 0.84 - 73.17). 513 biopsies were performed on patients under 18 years of age, including 70 biopsies on children under 5 years of age. In the total group of 296, the mean number of biopsies per patient was 3.08 ± 0.11 (median: 3; range: 1 - 8), with 91 patients biopsied only once (Table 3.3). In the 205 patients who had serial biopsies, the mean number of biopsies was 4.00 ± 0.11 (median: 4; range: 2 - 8). Serial biopsies were obtained a mean of 1.65 ± 0.04 years apart (median: 1.32, range: 0.19 - 6.65 years).
### Table 3.3. Number of Liver Biopsies Performed on 296 Patients

<table>
<thead>
<tr>
<th>No. of Biopsies</th>
<th>No. of Patients</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
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<tr>
<td>3</td>
<td>41</td>
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<td>33</td>
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<td>53</td>
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<td>20</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>296</strong></td>
</tr>
</tbody>
</table>

#### 3.4.1 Success at Obtaining Adequate Tissue

Table 3.4 summarizes the success at obtaining adequate tissue by percutaneous liver biopsy. From the entire series of 911 liver biopsies, sample weights of the portion of liver tissue used for iron quantitation were reported in 873 (95.8%) of biopsies, while histology reports were obtained for 894 (98.1%) of biopsies. Both iron and histology reports were obtained in 858 / 911 (94.2%) of biopsies. An iron report but no histology report was obtained in 15 cases, while a histology report but no iron report was obtained in 36 cases. Neither the iron report nor the histology reported could be found for only 2 biopsies.

Of the 858 biopsies that produced a complete set of reports, adequate tissue for both iron and histological analysis was obtained in 666 (77.6%) of these biopsies. However, only 17 biopsies, out of this group of 858 (2.0%), failed to acquire enough tissue for both accurate iron quantitation and definitive histological assessment. The remaining 175 (of the 858) biopsies, obtained adequate tissue for either iron or histological analysis. Therefore, 841/858 (98.0%) of these biopsies provided adequate tissue for either iron analysis or histological assessment or both.

From the total 873 iron samples with tissue weights reported, 736 (84.3%) were of adequate size for accurate biochemical iron quantitation, weighing $\geq 1.0$ mg, dry (Angelucci et al., 2000). The mean dry weight of these liver samples was $2.26 \pm 0.06$ mg (median: 1.97; range: 0.1 – 29.7 mg). The annual success rate at obtaining adequate tissue is reported in Table 3.4.
Table 3.4. Annual success rate at obtaining liver biopsy tissue adequate for iron quantitation and histological assessment at HSC and TGH.

<table>
<thead>
<tr>
<th>Year</th>
<th>Hosp.</th>
<th>No. of Bx's</th>
<th>No. (%) of Bx's</th>
<th>No. of Bx's</th>
<th>No. (%) of Bx's</th>
<th>No. of Bx's</th>
<th>No. (%) of Bx's</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>with HIC</td>
<td>adequate for HIC</td>
<td>with path.</td>
<td>with definitive</td>
<td>with both</td>
<td>adequate for HIC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>report</td>
<td>(≥1 mg, dry wt)</td>
<td>report</td>
<td>histology</td>
<td>reports</td>
<td>and HIC</td>
</tr>
<tr>
<td>1990</td>
<td>HSC</td>
<td>3</td>
<td>2 (100%)</td>
<td>3</td>
<td>3 (100%)</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
<tr>
<td></td>
<td>TGH</td>
<td>13</td>
<td>13 (76.9%)</td>
<td>13</td>
<td>12 (92.3%)</td>
<td>13</td>
<td>9 (69.2%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>15 (80.0%)</td>
<td>16</td>
<td>15 (93.8%)</td>
<td>15</td>
<td>11 (73.3%)</td>
</tr>
<tr>
<td>1991</td>
<td>HSC</td>
<td>3</td>
<td>3 (66.7%)</td>
<td>3</td>
<td>3 (100%)</td>
<td>3</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td></td>
<td>TGH</td>
<td>27</td>
<td>15 (55.6%)</td>
<td>23</td>
<td>23 (92.0%)</td>
<td>25</td>
<td>14 (56.0%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>30</td>
<td>17 (56.7%)</td>
<td>28</td>
<td>26 (92.9%)</td>
<td>28</td>
<td>16 (51.1%)</td>
</tr>
<tr>
<td>1992</td>
<td>HSC</td>
<td>22</td>
<td>15 (68.2%)</td>
<td>22</td>
<td>18 (81.8%)</td>
<td>22</td>
<td>14 (63.6%)</td>
</tr>
<tr>
<td></td>
<td>TGH</td>
<td>35</td>
<td>20 (60.6%)</td>
<td>35</td>
<td>25 (71.4%)</td>
<td>33</td>
<td>16 (48.5%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>57</td>
<td>35 (63.6%)</td>
<td>57</td>
<td>43 (75.4%)</td>
<td>55</td>
<td>30 (54.5%)</td>
</tr>
<tr>
<td>1993</td>
<td>HSC</td>
<td>26</td>
<td>19 (90.5%)</td>
<td>24</td>
<td>22 (91.7%)</td>
<td>19</td>
<td>15 (78.9%)</td>
</tr>
<tr>
<td>*</td>
<td>TGH</td>
<td>29</td>
<td>26 (100.0%)</td>
<td>29</td>
<td>22 (75.9%)</td>
<td>26</td>
<td>20 (76.9%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>55</td>
<td>47 (85.7%)</td>
<td>53</td>
<td>44 (83.0%)</td>
<td>45</td>
<td>35 (77.8%)</td>
</tr>
<tr>
<td>1994</td>
<td>HSC</td>
<td>24</td>
<td>15 (75.0%)</td>
<td>24</td>
<td>20 (83.3%)</td>
<td>20</td>
<td>15 (75.0%)</td>
</tr>
<tr>
<td>*</td>
<td>TGH</td>
<td>30</td>
<td>15 (75.0%)</td>
<td>29</td>
<td>20 (69.0%)</td>
<td>20</td>
<td>10 (50.0%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>54</td>
<td>40 (75.0%)</td>
<td>53</td>
<td>40 (75.5%)</td>
<td>40</td>
<td>25 (62.5%)</td>
</tr>
<tr>
<td>1995</td>
<td>HSC</td>
<td>75</td>
<td>71 (94.7%)</td>
<td>73</td>
<td>72 (98.6%)</td>
<td>73</td>
<td>68 (93.2%)</td>
</tr>
<tr>
<td>*</td>
<td>TGH</td>
<td>75</td>
<td>71 (94.7%)</td>
<td>73</td>
<td>72 (98.6%)</td>
<td>73</td>
<td>68 (93.2%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>99</td>
<td>96 (97.7%)</td>
<td>95</td>
<td>89 (93.7%)</td>
<td>93</td>
<td>79 (84.9%)</td>
</tr>
<tr>
<td>1996</td>
<td>HSC</td>
<td>85</td>
<td>68 (81.0%)</td>
<td>82</td>
<td>75 (91.4%)</td>
<td>81</td>
<td>61 (75.3%)</td>
</tr>
<tr>
<td>*</td>
<td>TGH</td>
<td>75</td>
<td>68 (81.0%)</td>
<td>82</td>
<td>75 (91.4%)</td>
<td>81</td>
<td>61 (75.3%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>111</td>
<td>109 (90.0%)</td>
<td>107</td>
<td>92 (86.0%)</td>
<td>105</td>
<td>75 (71.4%)</td>
</tr>
<tr>
<td>1997</td>
<td>HSC</td>
<td>71</td>
<td>666 (95.7%)</td>
<td>71</td>
<td>69 (97.2%)</td>
<td>70</td>
<td>65 (92.9%)</td>
</tr>
<tr>
<td>*</td>
<td>TGH</td>
<td>70</td>
<td>666 (95.7%)</td>
<td>71</td>
<td>69 (97.2%)</td>
<td>70</td>
<td>65 (92.9%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>131</td>
<td>126 (89.5%)</td>
<td>130</td>
<td>125 (96.2%)</td>
<td>125</td>
<td>93 (74.4%)</td>
</tr>
<tr>
<td>1998</td>
<td>HSC</td>
<td>73</td>
<td>666 (97.3%)</td>
<td>71</td>
<td>60 (84.5%)</td>
<td>71</td>
<td>58 (81.7%)</td>
</tr>
<tr>
<td>**</td>
<td>TGH</td>
<td>63</td>
<td>666 (94.1%)</td>
<td>63</td>
<td>63 (100%)</td>
<td>62</td>
<td>52 (83.9%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>137</td>
<td>136 (91.2%)</td>
<td>134</td>
<td>123 (91.8%)</td>
<td>133</td>
<td>110 (82.7%)</td>
</tr>
<tr>
<td>1999</td>
<td>HSC</td>
<td>69</td>
<td>666 (95.7%)</td>
<td>69</td>
<td>67 (97.1%)</td>
<td>69</td>
<td>64 (92.8%)</td>
</tr>
<tr>
<td>**</td>
<td>TGH</td>
<td>42</td>
<td>666 (78.6%)</td>
<td>42</td>
<td>41 (97.6%)</td>
<td>42</td>
<td>32 (76.2%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>111</td>
<td>111 (89.2%)</td>
<td>111</td>
<td>108 (97.3%)</td>
<td>111</td>
<td>96 (86.5%)</td>
</tr>
<tr>
<td>2000</td>
<td>HSC</td>
<td>65</td>
<td>666 (96.9%)</td>
<td>65</td>
<td>62 (95.4%)</td>
<td>64</td>
<td>59 (92.2%)</td>
</tr>
<tr>
<td>**</td>
<td>TGH</td>
<td>44</td>
<td>666 (84.1%)</td>
<td>45</td>
<td>45 (100%)</td>
<td>44</td>
<td>37 (84.1%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>110</td>
<td>108 (91.7%)</td>
<td>110</td>
<td>107 (97.3%)</td>
<td>108</td>
<td>96 (88.9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grand Total</td>
<td>911</td>
<td>873</td>
<td>736 (84.3%)</td>
<td>894</td>
<td>812 (90.8%)</td>
</tr>
</tbody>
</table>

* Biopsies performed using ultrasound-guidance at HSC.
** Biopsies performed using ultrasound-guidance at both HSC and TGH.
From the total 894 pathology reports collected, 812 (90.8%) samples were able to provide a definitive microscopic description of hepatic iron distribution, fibrosis, and portal inflammation.

3.4.2 Impact of Ultrasound

Blind (not ultrasound-guided) biopsies, performed before 1993 at our pediatric center and before 1998 at our adult center, obtained adequate tissue for both iron quantitation and histological assessment in 140 / 243 (57.6%) of biopsies (Figure 3.1). Of the 103 inadequate iron samples from these mainly 'blind' biopsies, 94 were from our adult center and 9 were from our pediatric center. After ultrasound-guidance became standard technique for liver biopsies, adequate tissue was obtained in 526 / 615 (85.5%) of biopsies, a statistically significant improvement from the blind technique (p<0.001). Of the 89 inadequate iron samples from ultrasound-guided biopsies, 27 were from our adult center and 62 were from our pediatric center.

Blind biopsies performed before 1993 at our pediatric center and before 1998 at our adult center, obtained adequate tissue for iron quantitation in 174 / 248 (70.2%) of cases (Figure 3.2). Of the 74 inadequate iron samples from blind biopsies, 66 were from our adult center and 8 were from our pediatric center. After ultrasound-guidance became standard technique for liver biopsies, adequate tissue was obtained in 562 / 625 (89.9%) of biopsies, a statistically significant improvement from the blind technique (p<0.001). Of the 63 inadequate iron samples from ultrasound-guided biopsies, 26 were from our adult center and 37 were from our pediatric center.

Blind biopsies acquired sufficient tissue for pathological analysis in 216 / 264 (81.8%) of cases. In comparison, ultrasound-guided biopsies were significantly more successful (p<0.001), capturing adequate tissue in 596 / 630 (94.6%) of cases (Figure 3.3). Of the 82 inadequate samples, 45 were from biopsies at our adult center, of which 43 were not ultrasound-guided. In comparison, only 5 of the 37 inadequate histology samples obtained at our pediatric center were from biopsies performed without ultrasound guidance.
Figure 3.1. Trends in the annual success at obtaining adequate liver tissue for BOTH hepatic iron quantitation and histological assessment, in haemoglobinopathy patients at the Hospital for Sick Children (HSC) and the Toronto General Hospital (TGH).

Note: For each of the hospitals, the numbers in parentheses indicate the number of biopsies that had reports for both iron concentration and histology.
Figure 3.2. Trends in the annual success at obtaining adequate tissue for hepatic iron quantitation, in hemoglobinopathy patients at the Hospital for Sick Children (HSC) and the Toronto General Hospital (TGH).

Figure 3.3. Trends in the annual success at obtaining adequate tissue for histological assessment, in hemoglobinopathy patients at the Hospital for Sick Children (HSC) and the Toronto General Hospital (TGH).
3.4.3 Morbidity/Complications and Mortality

All biopsies were performed without any major hemorrhagic complications requiring transfusion, and only seven minor complications. Five minor complications were associated with non-US-guided biopsies including renal puncture (n=1), soft-tissue biopsy (n=2), pulmonary effusion (n=1), and intolerable pain at the biopsy site (n=1). Only two minor complications resulted from US-guided biopsies, including a small anterior subcapsular hematoma (n=1) and shoulder tip pain (n=1). The two patients with minor pain complications were the only patients admitted for overnight observation, and relatively few patients required post-procedure pain management.

3.4.4 Cost-Analysis

The cost of a hepatic iron quantitation was divided into ultrasound-guided biopsy procedure costs, and biochemical iron assay costs (including sample storage and shipping). The total cost of determining the hepatic iron concentration from a sample of liver tissue obtained through ultrasound-guided biopsy was estimated at $244.22 (Table 3.5).

<p>| Table 3.5. Costs associated with hepatic iron quantitation of biopsy tissue. |
|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Item</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy needle</td>
<td>$45.00</td>
</tr>
<tr>
<td>Supplies (needles, syringes, etc)</td>
<td>$42.00</td>
</tr>
<tr>
<td>Ultrasound (-guidance)</td>
<td>$12.00</td>
</tr>
<tr>
<td>Medication</td>
<td>$10.00</td>
</tr>
<tr>
<td>Physician fee</td>
<td>$67.34 (OHIP fee)</td>
</tr>
<tr>
<td>Nurse and technologist fee</td>
<td>$50.00 (1 hour @ $25/hr each)</td>
</tr>
<tr>
<td><strong>SUBTOTAL</strong></td>
<td><strong>$226.34</strong></td>
</tr>
<tr>
<td>Sample storage and shipping</td>
<td>$6.00</td>
</tr>
<tr>
<td>Biochemical Iron Assay</td>
<td>$11.88</td>
</tr>
<tr>
<td><strong>SUBTOTAL</strong></td>
<td><strong>$17.88</strong></td>
</tr>
<tr>
<td><strong>GRAND TOTAL</strong></td>
<td><strong>$244.22</strong></td>
</tr>
</tbody>
</table>

The cost (OHIP rate) to measure the serum ferritin concentration from a blood sample sent to the hospital core lab is $12.48.
3.5 Discussion

Direct biochemical quantitation of the hepatic iron concentration of a liver tissue sample, based on the simple method originally described by Barry and Sherlock (1971), remains the gold standard technique for evaluating the total body iron store in iron loaded individuals (Olivieri and Brittenham, 1997). Percutaneous liver biopsy is a commonly used technique for obtaining a sample of liver tissue for biochemical iron quantitation.

In several hemoglobinopathies, patients characteristically accumulate iron in their bodies, either through increased iron absorption or through regular red blood cell transfusion therapy. Therefore, these patients require regular monitoring of body iron levels, in order to justifiably make adjustments in therapy (i.e. chelation). However, clinicians are still reluctant to adopt liver biopsies into their hemoglobinopathy programs, due to the perceived invasiveness of the procedure, which may expose the patient to unnecessary risks of significant morbidity and mortality. As a result, iron levels are often monitored using indirect indicators, commonly the serum ferritin concentration, which are much less reliable markers of iron overload.

The observations and results reported in this study confirm that liver biopsy is associated with an incredibly low risk of morbidity (<1%), and essentially no risk of mortality. In addition to providing the most accurate and reliable measure of body iron burden, liver biopsy provides the opportunity to assess liver histology, including fibrosis, cirrhosis, portal inflammation, hepatitis, and other liver pathology often secondary to iron overload. We have been able to successfully integrate regular liver biopsies into our practice, a testament to the patient’s tolerance of the procedure. Body iron levels have been monitored using serial biopsies in over 200 patients, with a mean interval between biopsies of approximately 20 months. In patients suspected or evidently non-compliant with chelation therapy, the biopsy interval may be abbreviated, while in those who are very compliant, the interval may be appropriately extended. Furthermore, our experience with liver biopsies has encouraged us to perform liver biopsies even in patients as young as 10 months old, when concerned about the rapid accumulation of transfusional iron overload (Section 4). Notably, all 15 biopsies performed on patients under 2 years of age, were well-tolerated and uncomplicated.
The primary objective of liver biopsy in hemoglobinopathy patients is to obtain an adequate sample for iron quantitation. Overall, since 1990 we report that liver biopsies were able to acquire an adequate iron sample in 84% of cases. Focusing on the last 3 years, when ultrasound-guidance was used consistently, over 90% of biopsies obtained an adequate sample for iron. Notably, biopsies that failed to produce an adequate iron sample, almost always managed to still obtain enough tissue for histological assessment. Therefore the biopsy cannot be considered a complete failure.

Since 1990, we report an overall complete success rate (adequate tissue for both assessments) of approximately 78% of biopsies, while 20% of biopsies were partially successful. Since 1996, a consistent yearly improvement in the complete success rate has been observed, from approximately 71% to 89% in the year 2000. Annually since 1998, we’ve achieved a total success rate above 80%, with a mean of approximately 86%, among the 358 biopsies performed. All remaining biopsies, however, were still able to provide either a histological assessment or reliable hepatic iron concentration.

Prior to 1993, several of our biopsies were performed without ultrasound guidance by our gastroenterology service. In 1993, the decision was made at our pediatric center, to have all our liver biopsies performed by an interventional radiologist using ultrasound guidance. The outcome of this decision was a significantly increased rate of successful biopsies at our pediatric center. A significant improvement in the rate of successful biopsies was also observed at our adult center following the introduction of ultrasound-guidance in 1998 (Figure 3.1).

Other factors that may improve the operators/interventionalists success in obtaining adequate tissue include experience with the biopsy technique, and the type of biopsy needle used. At our pediatric center, almost all of the liver biopsies performed since 1993 were by 2 staff interventional radiologists, and temporal increase in success rate may reflect their evolving experience.

Of particular interest is the peak in the rate of successful biopsies observed in 1993. Although at the pediatric center this has been suggested to be related to adopting ultrasound-guidance into the biopsy technique, this cannot explain the marked improvement at the adult center. A reasonable alternative explanation focuses on the enrollment of several hemoglobinopathy patients into a clinical trial, with their initial
study biopsies performed in 1993. For this study, instructions were sent to the biopsy departments at both centers, educating or reminding the operators/interventionalists about the amount of tissue that must be obtained. This may coincidentally highlight the effectiveness of communication between departments, and suggests that periodically reminding the biopsy centers about the amount of tissue required, may improve the success rate in a liver biopsy program.

Although impossible to properly investigate in this study, the amount of liver tissue sent to each laboratory for analysis does not depend entirely on the amount of tissue obtained by the operator/interventionalist. Inadequate liver samples may be generated as a result of improper handling and processing of the specimen, particularly in dividing the biopsy tissue obtained into a sample for iron quantitation and a sample for histology (ie. too much tissue sent to one lab and not enough to the other lab). Dividing the tissue in two equal portions does not guarantee that both laboratories receive adequate tissue samples, and may result in inadequate samples sent to both labs. If quantitative guidelines analogous to the 1.0mg, dry weight threshold for iron analysis (Angelucci et al., 2000), were established for the histology sample, the tissue obtained at biopsy could perhaps be weighed and divided appropriately, or when to send an entire specimen to only one of the two laboratories.

Morbidity and Mortality

Our results also confirm that sufficient hepatic tissue can be obtained safely from out-patient percutaneous liver biopsy. In this series, no significant complications or biopsy-related deaths occurred. Hemorrhagic complications continue to be the most common major complications associated with liver biopsies reported in the literature (Gilmore et al., 1995; Smith et al., 1995; Angelucci et al., 1995; Kliwer et al.,1999; Cadranel et al., 2000). However, improvements in ultrasound image quality may allow interventionists to better visualize the liver, avoiding vascular structures and more confidently guiding biopsy needles into the liver. To date, we have experienced no significant hemorrhagic complications requiring transfusion or hospital admission.
Cost-effectiveness of hepatic iron quantitation for monitoring iron overload

The estimated cost of performing a liver biopsy and obtaining a biochemically quantitated hepatic iron concentration was $244.22. Therefore, the cost of accurately monitoring body iron levels over any period of time would be $488.44, the cost of two biopsies and assays. In comparison, based on the mean interval of 20 months between biopsies in this series, the cost of monitoring body iron using monthly serum ferritin concentrations ($12.48) over the same interval would accumulate a cost of approximately $250.00, almost half of the cost of the quantitative technique. Nevertheless, the added expense may be justified by the optimal accuracy of the iron quantitation assay, and lack of reliability of serum ferritin as an indicator of iron overload. Furthermore, the liver biopsy also provides tissue that is analyzed histologically, at no added operational cost.

In conclusion, out-patient ultrasound-guided percutaneous core liver biopsy is a safe, effective, and efficient procedure for obtaining hepatic tissue for iron quantitation and histological analysis in patients with hemoglobinopathies, including thalassemia major and sickle cell disease. We are currently obtaining adequate tissue for both the accurate chemical quantitation of hepatic iron concentration and definitive histological assessment of liver tissue in approximately 90% of biopsies. The results of these biopsies are ideal for guiding clinicians making chelation therapy decisions. As ultrasound image quality continues to improve, interventional radiologists should become even better able to safely obtain adequate liver tissue. We believe that an ultrasound-guided core liver biopsy should be periodically performed on transfused hemoglobinopathy patients to accurately monitor body iron levels and assess the liver histology, and suggest that other clinicians incorporate serial liver biopsies into their hemoglobinopathy programs.

3.5.1 Limitations and Future Direction

The 1.0mg, dry weight threshold for the amount of tissue required to accurately quantify the hepatic iron concentration using the biochemical iron quantitation assay, has been well established in the literature (Angelucci, 2000). In comparison, no quantitative
threshold of tissue has been established for histological assessment. Investigators use a range of cut-off weights, tissue core lengths, and numbers of portal tracts in the tissue specimen, without justification. In this study, neither weight, nor size, nor the number of portal tracts, could be used. This was exclusively due to the inconsistency among either (1) interventionalists and surgeons in reporting the amount of tissue obtained, or (2) pathologists, in reporting the dimensions of the sample and number of portal tracts observed. Whenever a pathologist commented on the poor quality of a specimen, this was interpreted as an inadequate sample. However, whenever a pathologist provided a score for each of the histological categories, without commenting on the adequacy of the specimen, this was interpreted as being a definitively diagnostic biopsy (as it would be interpreted by most clinicians).

Guidelines must be defined outlining the proper amount of tissue that must be obtained for histological analysis. Furthermore, a method of weighing and quantitatively dividing the tissue into an iron sample and histology sample, before any is transferred to formalin, and for communicating with the interventionalist during the procedure when more tissue is needed, would be ideal.
Chapter 4

PROGRESSION OF BODY IRON LOADING IN TRANSFUSED PATIENTS WITH SICKLE CELL DISEASE AND THALASSEMIA

4.1 Background and Literature Review

Regular red blood cell transfusion therapy is life-sustaining in patients with severe thalassemia syndromes (Whipple and Bradford, 1936, Frumin et al., 1952, Ellis et al., 1954, Witzleben and Wyatt, 1961), and improves survival and reduces morbidity in patients with sickle cell disease (Powars et al., 1978; Emond et al., 1985; Wang et al., 1991; Styles et al., 1994; Pegelow, 1995; Olivieri, 1997; Adams, 1998; Vichinsky et al., 1998; Weatherall, 2000; Ohene-Frempong, 2001). The prophylactic strategy involves suppressing endogenous erythropoiesis and infusing healthy red blood cells containing normal adult hemoglobin (HbA). In thalassemia, maintaining near-normal pre-transfusion hemoglobins prevents hypoxia, hypersplenism, and the development of bone malformations due to bone marrow expansion, permitting normal growth and development throughout childhood (Piomelli et al., 1969; Piomelli et al., 1995; Cazzola, 1995; Cazzola, 1997; Olivieri and Brittenham, 1997; Rund, 2000). In sickle cell disease, reducing and maintaining the concentration of HbS at less than 30% of total circulating hemoglobin minimizes the sickling of red blood cells, the rigid, vaso-occlusive cells responsible for sickle cells crises. Although never confirmed by clinical trial, studies have suggested that maintaining the level of sickle hemoglobin at less than 30%, through prophylactic transfusion, effectively protects sickle cell patients at high risk of stroke (Wang et al., 1991; Pegelow et al., 1995; Cohen et al., 1992).

The major caveat of red cell transfusion therapy is the accumulation of iron in the body, due to the lack of appropriate physiological mechanisms of eliminating iron. Disease complications and early death have been associated with increased iron overload in regularly transfused patients with B-thalassemia (Brittenham et al., 1994; Olivieri, 1994), which has raised the concern about the effects of iron overload in patients with
sickle cell disease. However, the rate of accumulation of iron in these disorders has not been appropriately quantified. Without chelation therapy, regularly transfused patients with thalassemia accumulate toxic and potentially lethal amounts of iron by early adolescence, and rarely survive beyond the third decade of life (Engle et al., 1964; Brittenham et al., 1994; Olivieri et al., 1994).

Several clinicians have adopted liver biopsies into their hemoglobinopathy programs to obtain tissue for (1) biochemical hepatic iron quantitation to monitor body iron levels, and (2) histological assessment to detect and monitor the progression of liver disease. Normal individuals have hepatic iron concentrations approximately between 0.6 to 1.2 mg of iron per gram liver, dry weight (Brittenham et al., 1982; NEJM). Normal survival without developing any of the complications associated with iron overload, has been observed in heterozygotes for hereditary hemochromatosis, who maintain hepatic iron concentrations below 7 mg/g, dry weight (Cartwright et al., 1979; NEJM). Furthermore, from studies of homozygous hemochromatosis, exceeding the 7mg/g threshold is associated with an increased risk of iron-induced complications including hepatic fibrosis and diabetes (Niederau et al., 1985; NEJM and 1996; Gastroenterology). Elevated hepatic iron concentrations, beyond 15mg/g, have been associated with an increased risk for iron-induced cardiac disease and early death (Olivieri et al., 1992; Am J Hem). Of particular note is that the goal of iron-chelation therapy is to maintain liver irons below 7 mg/g, but not lower than 3.2 mg/g beyond which the issue of chelator-induced complications becomes a concern. Through body iron quantitation, iron overload can be accurately detected, and the initiation of chelation therapy can be rationally based.

Despite the reliability of hepatic iron quantitation, the most common method of assessing and monitoring body iron levels focuses on the serum ferritin concentration. However, there is a growing awareness that these measures may not be accurate enough to be a reliable indicator of iron overload in both thalassemia and sickle cell disease (DeVirgillis, 1980; Worwood et al., 1980; Brittenham et al., 1993; Nielson et al., 1995; Piga et al., 1999; Vieira et al., 1999; Harmatz et al., 2000).
In this study, we examined the extent of iron overload, as well as the presence of liver injury, in children with thalassemia and sickle cell disease within the initial years of regular transfusion therapy. The objectives of the study were to identify:

1. The rate of iron accumulation and how quickly iron accumulates beyond thresholds of increased risk of iron toxicity in these patients.
2. The incidence and degree of liver damage associated with elevated body iron levels in these patients.
3. The similarities and differences in iron accumulation between the two disorders.
4. The reliability of serum ferritin concentration as a measure of body iron levels.

The results should highlight the requirement for quantitation of body iron burden, due to the lack of precision of surrogate markers, such as serum ferritin, in estimating iron overload. Through body iron quantitation, iron overload can be accurately detected and the initiation of chelation therapy can be rationally based.
4.2 Experimental Design

4.2.1 Sample Population

The medical records of all regularly transfused Sickle Cell Disease (SCD) and Thalassemia major (TM) patients managed through the Toronto Hemoglobinopathy Program of the University of Toronto, who had hepatic iron quantitated prior to exposure to chelation therapy, were reviewed. This study included 40 patients, 18 with Sickle Cell Disease and 22 with Thalassemia major.

4.2.1.1 Inclusion/Exclusion Criteria

All patients with incomplete blood transfusion records were excluded from this study. Complete transfusion records included the dates of all transfusions, the patient’s weight on the day of each transfusion, the total volume and hematocrit of each blood transfusion. Only patients who had hepatic iron concentration quantitatively measured prior to chelation therapy were included in this study. Strict adherence to these criteria limited our sample size to 18 SCD patients (4 males, 14 females) and 22 TM patients (9 males, 13 females).

4.2.1.2 Indications for Regular Blood Transfusion Therapy

The 22 patients with Thalassemia major included in this study began regular blood transfusion therapy at the age of 1.1 ± 0.3 (range: -0.2 – 4.8) years, maintaining baseline hemoglobin concentrations of approximately 9.5 g/dL through transfusions every 25 ± 0.7 (range 15 – 31) days, in order to survive and achieve normal growth and development. The indications for chronic red cell transfusion therapy in patients with sickle cell disease are expanding. In this study, our 18 sickle cell patients were introduced to transfusion therapy at the age of 7.8 ± 1.1 (range: 0.9 – 15.6) years, in response to central nervous system stroke (n=9), the identification of elevated transcranial...
doppler velocities through screening (n=4), acute splenic sequestration (n=3), and severe vaso-occlusive crises (n=2). Sickle cell patients were transfused at approximately 30 ± 1.3 (range: 18 – 39) day intervals, in order to maintain sickle hemoglobin concentrations at less than 30% of the total circulating hemoglobin.

4.2.2 Red Blood Cell Transfusion Therapy

The number of months of transfusion therapy was recorded, as was the total volume of red cells that patients were transfused per kilogram body weight measured on the day of biopsy. Assuming that 1mL of red cells contains 1mg of iron, the volume of red cells transfused also represents the total mg of iron these patients were transfused (per kg body weight on the day of biopsy).

4.2.3 Liver Biopsy and Hepatic Iron Concentration

Measurement of hepatic iron concentration, or HIC, provides the most quantitative assessment of body iron burden (Pippard, 1989). Between November 1992 and March 2000, we obtained an initial measure of hepatic iron concentration in all 40 patients, before they received any chelation therapy. In the 18 SCD patients and in 19 TM patients, HIC was obtained through biochemical digestion and atomic absorption spectrophotometry of liver biopsy tissue. Liver biopsies were performed by an interventional radiologist using ultrasound-guidance (described in section 1.2.2.3) in 35 of the 37 cases. In the two remaining cases, liver biopsy was performed in association with another procedure in the operating room. Of particular note, is that 34 of the 37 tissue samples analyzed were >1mg in dry weight, a recently reported threshold for optimal accuracy of HIC measurements from biopsy tissue (Angelucci et al., 2000; NEJM). Hepatic iron concentration in the remaining three TM patients was obtained using SQUID magnetic susceptometry (described in section 1.2.2.4). All hepatic iron concentrations are reported in milligrams of iron per gram of liver tissue, dry weight (mg/gdw).
4.2.3.1 Hepatic Iron Concentration Biochemical Assay

All of the biopsy samples for iron quantitation obtained from the patients in this study, were sent to one of two iron labs for the biochemical HIC assay.

Cleveland (Brittingham) Assay for Hepatic Iron Quantitation

A chemical method described by Overmoyer et al. in 1987, which is based on a modification to the technique originally described by Wixom et al. in 1980. The Overmoyer technique measures the hepatic non-heme iron content (the contribution of heme iron in the liver is considered negligible in the presence of iron excess). Percutaneous biopsy samples are homogenized and diluted to a final tissue concentration of 1%. 200mL of this 1% homogenate is mixed with 70μL of 12N hydrochloric acid, incubated at 90°C for 1 hour, then left to cool to room temperature. After adding 140μL of 1.6M trichloracetic acid and left for 20 minutes, samples are centrifuged for 5 minutes at 10000g. 300μL of the supernatant is then added to 450μL of chromagen, and after 15 minutes the iron content is measured using 535nm atomic absorption spectrophotometry, and expressed in micrograms of iron per gram of liver tissue, wet weight.

Dundee, Scotland (Pippard) Assay for Hepatic Iron Quantitation

The other 4 biopsy samples were assayed for hepatic iron quantitation in July 1999 or later using a chemical assay based on the technique originally described by Barry and Sherman in 1971. A thorough description of the HIC assay used for these 4 samples is reported elsewhere (Pippard, 1983). Briefly, a tissue sample is oven-dried at 120°C until it reaches a constant weight. The tissue is then digested or homogenized using a concentrated mixture of sulfuric and nitric acid, and heating gently for 15 minutes. The residue is then cooled, hydrogen peroxide added, and then reheated. The solution becomes clear, and is then cooled and iron-free water is added. This solution is mixed and the absorbance at 535nm is measured and compared to a standard. Absorbance is directly proportional to HIC, which is reported in mgFe/g liver, dry weight.
4.2.4 Serum Ferritin Concentration

Serum ferritin concentrations were also obtained from each patient, to analyze serum ferritin trends between the start of transfusions and the initial liver biopsy. The SFC obtained on or nearest to the day of biopsy was also recorded to determine the correlation between SFC and hepatic iron concentration.

4.2.4.1 Serum Ferritin Concentration Assay

The serum ferritin assay at our institution has changed twice since 1990. Prior to March 1994, SFC was assayed using a radiometric 2-site sandwich immunoassay (Ramco Fer-Iron II kit). The amount of radiolabel bound to ferritin was measured in a gamma counter, and was directly proportional to the concentration of ferritin in a serum sample. Since March 1994, SFC has been assayed using a magnetic separation chemiluminimetric 2-site sandwich enzyme immunoassay. However between March 1994 and June 1997, the Bayer ACS:180™ system was used, and in June 1997 the hospital switched to a Bayer Technicon Immuno 1® system. Both systems are similar, with a direct relationship between the ferritin in a sample and the relative light units detected by the lab analyzer.

4.2.5 HIC and SFC Thresholds of Increased Risk

Hepatic iron concentration thresholds of increased risk have been well established through a collection of studies using cohorts of patients with thalassemia and hereditary hemochromatosis (Cartwright, 1979; Brittenham, 1994; Olivieri, 1995; Niederau, 1996; Olivieri, 1998; Telfer, 1999). Therefore, in our hemoglobinopathy patients, including those in this study, hepatic iron concentrations below 7 mg/gdw are considered to be safe, while exceeding the 7 mg/gdw threshold is associated with increased risk of iron-related complications including liver disease and various endocrinopathies. Furthermore, hepatic iron concentrations exceeding 15 mg/gdw are associated with a heightened risk of cardiac disease and early death.
Serum ferritin concentration thresholds of increased risk have not been appropriately established. However, studies have suggested that concentrations exceeding 2500 ng/ml may indicate an unsafe level warranting chelation therapy (Olivieri et al., 1994; Olivieri et al., 1998). More recently, it has been suggested that serum ferritin concentrations below 1500 ng/ml reflect safe body iron levels, indicating that chelation therapy may be appropriately delayed (Ballas, 2001).

In order to assess the reliability of serum ferritin concentration as an indicator of iron overload, we counted the number of patients with serum ferritin concentrations above the “suggested” SFC thresholds, with corresponding hepatic iron concentrations below the 7mg/gdw threshold of increased risk. These serum ferritin concentrations were identified as “false-positive”, as they would inaccurately indicate iron overload beyond safe thresholds. “False-negative” serum ferritin concentrations were also counted, defined as an SFC below the “suggested” threshold, while the corresponding HIC was above the 7mg/gdw threshold of increased risk of complications. These SFCs would have failed to identify iron overload beyond safe thresholds.

4.2.6 Histological Analysis of Hepatic Fibrosis, Inflammation and Iron Distribution

Histological analysis of all liver biopsy tissue, using hematoxylin-eosin, Masson’s trichrome, and Perls’ iron stained sections of all samples, was obtained from the patient’s medical records. A histological evaluation of iron distribution was also performed by a single pathologist, blinded to the patient’s identity and diagnosis. Hepatic fibrosis and portal inflammation were graded separately using the modified Scheuer index, with a range between 0 (no abnormality) and 4 (cirrhosis or severe portal inflammation, respectively) (Ludwig, 1993; Desmet et al., 1994). The pattern of iron staining, assessing iron distribution and intensity, was evaluated using the grading system described by Deugnier et al (1992). This system scores the amount and intensity of iron deposited within each of the three microcirculatory zones of hepatocytes (range 0 to 12, for a total hepatocytic iron score out of 36), sinusoidal (Kupffer) cells (range 0 to 4, for a total sinusoidal iron score out of 12), and portal tracts (range: 0 to 4, for a total portal iron
score out of 12). The larger maximum score in hepatocytic iron reflects the 3:1 ratio of the number of hepatocytes to Kupffer cells.

4.3 Statistical Analysis

4.3.1 Group comparison (Two-sample t-test and Fisher Exact test)

The two-sample t-test (without the assumption of equality of variances) was used to reveal significant differences between the two groups when comparing means and distributions (Dixon, 1985; Pagano, 2000). Parameters reporting frequencies or ratios were compared using the Fisher exact test. All tests were two-tailed, using a significance level of 0.05.

4.3.2 Pearson’s Correlation and Regression Analysis

The linear relationships between the hepatic iron concentration and (1) the duration of transfusion therapy, (2) the volume of transfused red cells per kilogram body weight at biopsy, and (3) the serum ferritin concentration, were assessed using Pearson's correlation analysis and linear regression analysis. The coefficient of determination was used to estimate the proportion of variation in hepatic iron concentration that could be accounted for by each of the 3 variables listed above.

4.3.3 F-Test for Equality of Regression Lines Across Groups

The F test for equality of regression lines across groups was used to determine if the regression lines (slopes and intercepts) are similar. Should the F-test reveal a non-significant difference of the regression lines, the conclusion would be that the two groups have similar regression equations, and the data from the two groups could be pooled for further analyses (Dixon, 1985; Zar, 1984).
4.3.4 Prediction Intervals

Linear regression analysis was also used to define 95% and 80% confidence intervals for predicting both the mean and individual hepatic iron concentrations at selected months and volumes of transfusion therapy, and selected serum ferritin concentrations.

The standard error of the predicted values for the mean HIC represents the potential variability we would observe in other samples of "n" patients after a particular number of months or volume of transfusions, or with a particular SFC. Alternatively, instead of predicting the mean HIC, we may prefer to predict the HIC for an individual new member of the population. The standard error for these predicted values is not the same as the standard error used when estimating mean HIC. When considering an individual HIC, we have an extra source of variability to account for, which is the variation of the HIC values themselves around the predicted mean. (There is a different formula with an added term). Therefore, because of the extra source of variability, the limits on a predicted individual value of HIC are wider than the limits on the predicted mean HIC.
4.4 Results

We studied 18 patients (4 males and 14 females) with Sickle Cell Disease (SCD) and 22 patients (9 males and 13 females) with Thalassemia major (TM). The characteristics of these 40 patients, and similarities between the two groups are shown in Table 4.1. Within the SCD group, regular red cell transfusion therapy began at a mean ± SEM age of 7.8 ± 1.1 years (range, 0.9 to 15.6 years), and patients were biopsied at the age of 9.2 ± 1.1 years (range, 2.2 to 17.9 years) without any complications. Within the TM group, regular red cell transfusion therapy began at a significantly younger mean age of 1.1 ± 0.3 years (range, -0.2 [pre-natal intrauterine transfusion] to 4.8 years) (p=0.0002). Furthermore, TM patients were biopsied at a significantly younger mean age of 2.6 ± 0.3 years (range, 0.8 to 5.9 years) (p=0.0002) without any complications. However, the total volume of transfused red blood cells per kilogram body weight at biopsy, and the duration of transfusion therapy, in the SCD group, 143.9 ± 8.6 mL/kg (range, 95.0 to 233.9 mL/kg) over 15.6 ± 1.2 months (range, 9.6 to 26.4 months), and in the TM group, 168.5 ± 15.4 mL/kg (range, 95.0 to 233.9 mL/kg) over 16.8 ± 1.2 months (range, 8.4 to 36.0 months), were both statistically similar (p=0.14 and p=0.39, respectively).

4.4.1 Indicators of Iron Accumulation

The characteristics of iron accumulation observed in these 40 SCD and TM patients are shown in Table 4.2. All 18 liver biopsy samples in the SCD group were adequate for precise iron quantitation, weighing greater than 1.0mg, dry weight (Angelucci et al., 2000). The mean hepatic iron concentration was 8.7 ± 0.9 mg/g, dry weight (range, 2.8 to 16.9 mg/g). HIC exceeded the 7mg/g threshold for iron-induced complications in 9 patients (50.0%), and in one patient (11.1%) also exceeded the 15mg/g threshold for iron-induced cardiac disease and premature death. The mean SFC obtained within 40.1 ± 15.0 days (range, 0 to 231 days) of the liver biopsy measured 2004.6 ± 205.5 ng/ml (range, 1087.0 to 4411.9 ng/ml). Pre-transfusion SFC measured 279.3 ± 71.4 ng/ml (range, 25.5 to 626.6 ng/ml).
Table 4.1. Patient Characteristics of the Regularly Transfused Patients with Sickle Cell Disease and Thalassemia major included in this study

<table>
<thead>
<tr>
<th></th>
<th>Sickle Cell Disease</th>
<th>Thalassemia Major</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Range)</td>
<td>(Range)</td>
<td></td>
</tr>
<tr>
<td>No. of patients</td>
<td>18</td>
<td>22</td>
<td>0.3119</td>
</tr>
<tr>
<td>Female sex (no. of patients)</td>
<td>14</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Age at start of regular transfusions (years)</td>
<td>7.8 ± 1.1</td>
<td>1.1 ± 0.3</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>(0.9 – 15.6)</td>
<td>(-0.2 – 4.8)</td>
<td></td>
</tr>
<tr>
<td>Age at liver biopsy / SQUID (years)</td>
<td>9.2 ± 1.1</td>
<td>2.6 ± 0.3</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>(2.2 – 17.9)</td>
<td>(0.8 – 5.9)</td>
<td></td>
</tr>
<tr>
<td>Duration of regular transfusions prior to biopsy (months)</td>
<td>15.6 ± 1.2</td>
<td>16.8 ± 1.2</td>
<td>0.3192</td>
</tr>
<tr>
<td></td>
<td>(9.6 – 26.4)</td>
<td>(8.4 – 36.0)</td>
<td></td>
</tr>
<tr>
<td>Total volume of RBC transfused pre-biopsy (mL)</td>
<td>4449.6 ± 501.0</td>
<td>2279.2 ± 268.5</td>
<td>0.0074</td>
</tr>
<tr>
<td></td>
<td>(1237.7 – 8554.0)</td>
<td>(659.3 – 5938.8)</td>
<td></td>
</tr>
<tr>
<td>Volume of RBCs transfused pre-biopsy (mL/kg at biopsy)</td>
<td>143.9 ± 8.6</td>
<td>168.5 ± 15.4</td>
<td>0.1408</td>
</tr>
<tr>
<td></td>
<td>(95.0 – 233.9)</td>
<td>(103.9 – 390.7)</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.2. Characterization of Iron Accumulation Before Starting Chelation Therapy

<table>
<thead>
<tr>
<th></th>
<th>Sickle Cell Disease</th>
<th>Thalassemia Major</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>18</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Initial HIC (mg/g, dry weight)</td>
<td>8.7 ± 0.9 (2.8 – 16.9)</td>
<td>9.8 ± 1.1 (2.8 – 22.4)</td>
<td>0.53</td>
</tr>
<tr>
<td>No. of patients with HIC &gt; 7mg/g, dry weight</td>
<td>9 / 18 (50.0%)</td>
<td>16 / 22 (72.7%)</td>
<td>0.19</td>
</tr>
<tr>
<td>No. of patients with HIC &gt; 15mg/g, dry weight</td>
<td>1 / 18 (5.6%)</td>
<td>3 / 22 (13.6%)</td>
<td>0.61</td>
</tr>
<tr>
<td>No. of patients with fibrosis</td>
<td>5 / 18 (27.8%)</td>
<td>6 / 17* (35.3%)</td>
<td>0.72</td>
</tr>
<tr>
<td>No. of patients with portal inflammation</td>
<td>4 / 18 (22.2%)</td>
<td>7 / 17* (41.2%)</td>
<td>0.29</td>
</tr>
<tr>
<td>Initial (baseline) SFC (ng/ml)</td>
<td>279.3 ± 71.4 (25.5 – 626.6)</td>
<td>629.3 ± 138.6 (93.7 – 3160.0)</td>
<td>0.044</td>
</tr>
<tr>
<td>Days between start of regular transfusions and initial SFC</td>
<td>-66.8 ± 30.0 (-415 – 25)</td>
<td>104.8 ± 24.3 (-2 – 390)</td>
<td>0.0001</td>
</tr>
<tr>
<td>SFC nearest to biopsy (ng/ml)</td>
<td>2004.6 ± 205.5 (1087.0 – 4411.9)</td>
<td>1498.6 ± 184.4 (741.9 – 4373.0)</td>
<td>0.17</td>
</tr>
<tr>
<td>Days between biopsy and nearest SFC (days)</td>
<td>40.1 ± 15.0 (0 – 231)</td>
<td>17.9 ± 4.7 (0 – 67)</td>
<td>0.13</td>
</tr>
<tr>
<td>Total Iron Score (/60)</td>
<td>21.2 ± 1.8 (12 – 37)</td>
<td>19.5 ± 2.4** (6 – 44)</td>
<td>0.57</td>
</tr>
<tr>
<td>Hepatocyte Iron Score (/36)</td>
<td>15.5 ± 1.3 (6 – 27)</td>
<td>13.7 ± 1.8** (3 – 30)</td>
<td>0.44</td>
</tr>
<tr>
<td>Kupffer Cell Iron Score (/12)</td>
<td>4.6 ± 0.4 (3 – 9)</td>
<td>4.0 ± 0.3** (3 – 7)</td>
<td>0.24</td>
</tr>
<tr>
<td>Portal Tract Iron Score (/12)</td>
<td>1.1 ± 0.2 (0 – 3)</td>
<td>1.8 ± 0.4** (0 – 7)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Adequate tissue for histology available in 17/22 patients (2 samples inadequate; no tissue obtained in 3 patients who had SQUI)

** Tissue for scoring iron available in 19/22 thalassemia patients (no tissue obtained in 3 patients who had SQUI)
In three of the 22 TM patients, hepatic iron concentrations were measured using SQUID magnetic susceptometry. Of the remaining 19 HICs, 16 biopsy samples weighed greater than 1.0mg, dry weight (Angelucci et al., 2000). The remaining 3 biopsy samples weighed greater than 0.6mg, dry weight, an earlier reported threshold for optimal accuracy (Olynyk et al., 1994). The mean hepatic iron concentration was 9.8 ± 1.1 mg/g, dry weight (range, 2.8 to 22.4 mg/g). HIC exceeded the 7mg/g threshold for iron-induced complications in 16 patients (72.7%), and in three patients (13.6%) also exceeded the 15mg/g threshold for iron-induced cardiac disease and premature death. The mean SFC obtained within 17.9 ± 4.7 days (range, 0 to 67 days) of the liver biopsy measured 1498.6 ± 184.4 ng/ml (range, 741.9 to 4373.0 ng/ml). Pre-transfusion SFC measured 629.3 ± 138.6 ng/ml (range, 93.7 to 3160.0 ng/ml).

A significant correlation between the number of months of regular transfusion therapy and hepatic iron concentration was observed in both the SCD group (r=0.69, p<0.005) and the TM group (r=0.76, p<0.005) (Figure 4.1). The correlation between the volume of red cells transfused per kilogram body weight at biopsy and hepatic iron concentration was also significant, but slightly weaker in the SCD group (r=0.65, p<0.005) and slightly stronger in the TM group (r=0.81, p<0.005) (Figure 4.2). In the TM group, a significant correlation between the serum ferritin concentration nearest to the biopsy and hepatic iron concentration was observed (r=0.61, p<0.005). However, in the SCD group, a much weaker and not statistically significant correlation was revealed between the serum ferritin concentration and the hepatic iron concentration (r=0.11, p=0.669) (Figure 4.3).

The reliability of serum ferritin as an indicator of iron overload was also analyzed by revealing how often the clinical interpretation of SFC matched the clinical interpretation of HIC (Tables 4.3a,b). Using the 2500 ng/mL “suggested” SFC threshold, 8 / 18 (44.4%) of the SFCs in sickle cell patients may have misguided clinicians (1 false-positive and 7 false-negative). In the TM group, 15 / 22 (68.2%) SFCs may have been misleading (1 false-positive and 14 false-negative). Using the more recently suggested 1500 ng/mL SFC threshold, 12 / 18 (66.6%) of the SFCs in sickle cell patients may have misguided clinicians (8 false-positive and 4 false-negative), while 14 / 22 (63.6%) of the SFCs may have been misleading in the TM group (3 false-positive and 11 false-negative).
Figure 4.1. Correlation between the Months of Transfusion Therapy and the Hepatic Iron Concentration in 18 Patients with Sickle Cell Disease (red) and 22 Patients with Thalassemia (blue). Shaded areas define the 95% confidence intervals for predicting an individual's HIC based on the duration of transfusion therapy.

From clinical experience with patients who are heterozygous for hemochromatosis, hepatic iron concentrations of approximately 3.2 to 7 mg of iron per gram of liver, dry weight (indicated in yellow), is associated with normal survival without complications of iron overload (Cartwright et al., 1979). Concentrations exceeding this range are associated with an increased risk of complications of iron overload (indicated in blue) (Niederau et al., 1996). From clinical experience with thalassemia major patients, concentrations exceeding 15 mg of iron per gram liver, dry weight, is associated with an increased risk of cardiac disease and early death (Brittenham et al., 1994). In the present study, hepatic iron concentrations exceeding 7 mg of iron per gram of liver, dry weight, were observed as early as 11.6 and 8.1 months following initiation of regular transfusions, in the sickle cell group and thalassemia group, respectively. However, the weakness in this correlation is evident as hepatic iron concentrations below 7 mg of iron per gram of liver, dry weight, are observed after as much as 16.8 months of transfusions in sickle cell disease, and as much as 19.4 months of transfusions in thalassemia. Hepatic iron concentrations exceeding 15 mg of iron per gram of liver, dry weight, were also observed in one sickle cell patient, and four thalassemia patients, after 18 to 36 months of transfusion therapy.
Figure 4.2. Correlation between the Total Volume of Transfused Red Blood Cells per Kilogram Body Weight at Biopsy and the Hepatic Iron Concentration in 18 Patients with Sickle Cell Disease (red) and 22 Patients with Thalassemia (blue). Shaded areas define the 95% confidence intervals for predicting an individual's HIC based on the total volume of transfused RBCs received.

In patients with sickle cell disease or thalassemia, hepatic iron concentrations exceeded the 7 mg of iron per gram of liver, dry weight, threshold of increased risk of complications of iron overload, after as little as 125 mL or 104 mL of red cells per kg body weight at biopsy, respectively. However, only moderate correlation was observed between the total volume of transfused red cells per kg body weight at biopsy and hepatic iron concentration, in both patient groups. Reflective of the high variability among hepatic iron concentrations in both groups, are the observations of concentrations below 7 mg of iron per gram of liver, dry weight (associated with normal survival without complications of iron overload), measured in one sickle cell and one thalassemia patient after as much as 161 mL and 150 mL of red cells per kilogram, respectively.
SFC does not correlate well with HIC in Thalassemia major and Sickle Cell Disease. In the Thalassemia group, the lowest SFC to exceed the 7 mg/g, dry weight, threshold of increased risk of complications was an SFC of 745.2 ng/mL, which corresponded with an HIC of 7.99 mg/g, dry weight. Meanwhile, an SFC of 2763.5 ng/mL was measured in a patient with an HIC of only 5.0 mg/g, dry weight. This is an over 2000 ng/mL range in SFC associated with the 7 mg/g threshold making it impossible to define SFC thresholds that corresponds with the established HIC thresholds of increased risk. In Sickle Cell Disease a much poorer and statistically insignificant correlation was observed. HIC exceeded the 7 mg/g, dry weight, threshold of increased risk of complications at SFCs as low as 1087 ng/mL, while SFCs as high as 4411.9 ng/mL corresponded with an HIC of 5.5 mg/g, dry weight (safely below the 7 mg/g threshold). This wide variation indicates that SFC is a clinically unreliable indicator of transfusional iron overload in both sickle cell disease and thalassemia major.
Table 4.3a. Reliability of SFC as an indicator of iron overload in 18 patients with sickle cell disease and 22 patients with thalassemia. (Based on a “Suggested” 1500ng/mL Threshold of Increased Risk)

<table>
<thead>
<tr>
<th></th>
<th>SCD (n)</th>
<th>Thal (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td># Patients with HIC &gt; 7mg/gdw</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td># Patients with SFC &lt; 1500 ng/mL</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td># Patients with HIC &gt; 7mg/gdw, SFC &lt;1500 ng/mL</td>
<td>4</td>
<td>11</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(False-negative – Chelation would be inappropriately delayed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td># Patients with HIC &lt; 7mg/gdw</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td># Patients with SFC &gt; 1500 ng/mL</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td># Patients with HIC &lt; 7mg/gdw, SFC &gt;1500 ng/mL</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(False-positive – Chelation would be prematurely initiated)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3b. Reliability of SFC as an indicator of iron overload in 18 patients with sickle cell disease and 22 patients with thalassemia. (Based on a “Suggested” 2500ng/mL Threshold of Increased Risk)

<table>
<thead>
<tr>
<th></th>
<th>SCD (n)</th>
<th>Thal (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td># Patients with HIC &gt; 7mg/gdw</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td># Patients with SFC &lt; 2500 ng/mL</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td># Patients with HIC &gt; 7mg/gdw, SFC &lt;2500 ng/mL</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(False-negative – Chelation would be inappropriately delayed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td># Patients with HIC &lt; 7mg/gdw</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td># Patients with SFC &gt; 2500 ng/mL</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td># Patients with HIC &lt; 7mg/gdw, SFC &gt;2500 ng/mL</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(False-positive – Chelation would be prematurely initiated)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4.2 Prediction Intervals

95% and 80% confidence intervals for predicting hepatic iron concentration were calculated using linear regression analyses. Tables 4.4a,b and 4.5a,b use selected months of transfusion therapy in SCD and TM, respectively. Similarly, Tables 4.6a,b and 4.7a,b use selected transfusional volumes, and tables 4.8a,b and 4.9a,b use selected serum ferritin concentrations.

4.4.3 Liver Iron Distribution

In patients with sickle cell disease, a mean \( \pm \) SEM of 72.7 \( \pm \) 2.3 % of the total iron was deposited in the hepatocyte while 22.2 \( \pm \) 1.7 % was deposited in the Kupffer cells, and 5.1 \( \pm \) 1.0 % was deposited in the portal tracts. In thalassemia, 67.2 \( \pm \) 2.5 % of the total iron was deposited in the hepatocyte, 25.1 \( \pm \) 3.0 % was deposited in the Kupffer cells, and 7.7 \( \pm \) 1.4 % was deposited in the portal tracts (Figure 4.5).

In patients with sickle cell disease, the relative deposition of iron within parenchymal and reticulo-endothelial cells was similar; the hepatocyte iron score was 43.1 \( \pm \) 3.7 % of the maximum possible score, compared to a mean Kupffer cell score of 38.4 \( \pm \) 3.5 % of the maximum possible score \( (p=0.371) \). Similarly, in patients with thalassemia, the relative deposition of iron within parenchymal and reticulo-endothelial cells was similar; mean hepatocyte iron score was 38 \( \pm \) 5 %, and mean Kupffer cell score was 33 \( \pm \) 3 %, of the maximum possible score \( (p=0.403) \). Therefore, no significant difference was apparent in the relative deposition of iron within the hepatocyte and Kupffer cell in patients with either sickle cell disease or thalassemia, while the pattern of iron distribution did not appear to be significantly different between these groups of patients.

Microscopic assessment of biopsy tissue also identified a distinct periportal-to-centrilobular hepatocyte iron concentration gradient, in patients with sickle cell disease and thalassemia. No similar Kupffer cell gradient was observed.
### Table 4.4a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Numbers of Months of Transfusion Therapy in Sickle Cell Disease

<table>
<thead>
<tr>
<th>Months Transfused</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.54</td>
<td>7.56</td>
</tr>
<tr>
<td>15</td>
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<td>14.26</td>
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<td>25</td>
<td>11.37</td>
<td>19.36</td>
</tr>
<tr>
<td>30</td>
<td>12.99</td>
<td>24.62</td>
</tr>
<tr>
<td>35</td>
<td>14.57</td>
<td>29.92</td>
</tr>
</tbody>
</table>

### Table 4.4b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Numbers of Months of Transfusion Therapy in Sickle Cell Disease

<table>
<thead>
<tr>
<th>Months Transfused</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.47</td>
<td>6.63</td>
</tr>
<tr>
<td>15</td>
<td>7.55</td>
<td>9.43</td>
</tr>
<tr>
<td>20</td>
<td>10.46</td>
<td>13.40</td>
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<tr>
<td>25</td>
<td>12.84</td>
<td>17.89</td>
</tr>
<tr>
<td>30</td>
<td>15.13</td>
<td>22.48</td>
</tr>
<tr>
<td>35</td>
<td>17.40</td>
<td>27.09</td>
</tr>
</tbody>
</table>

### Table 4.5a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Numbers of Months of Transfusion Therapy in Thalassemia major

<table>
<thead>
<tr>
<th>Months Transfused</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.27</td>
<td>8.33</td>
</tr>
<tr>
<td>15</td>
<td>7.20</td>
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<td>20</td>
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<td>12.74</td>
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<tr>
<td>25</td>
<td>11.52</td>
<td>15.71</td>
</tr>
<tr>
<td>30</td>
<td>13.19</td>
<td>18.91</td>
</tr>
<tr>
<td>35</td>
<td>14.77</td>
<td>22.21</td>
</tr>
</tbody>
</table>

### Table 4.5b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Numbers of Months of Transfusion Therapy in Thalassemia major

<table>
<thead>
<tr>
<th>Months Transfused</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.01</td>
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<tr>
<td>15</td>
<td>7.76</td>
<td>9.71</td>
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<td>12.17</td>
</tr>
<tr>
<td>25</td>
<td>12.28</td>
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<tr>
<td>35</td>
<td>16.13</td>
<td>20.85</td>
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</tbody>
</table>

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### Table 4.6a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Volumes of Transfused Red Blood Cells per Kilogram Body Weight at Biopsy in SCD

<table>
<thead>
<tr>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
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</thead>
<tbody>
<tr>
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<td>3.09</td>
<td>8.03</td>
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<td>0</td>
<td>12.57</td>
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<tr>
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<td>10.70</td>
<td>150</td>
<td>2.38</td>
<td>15.88</td>
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<tr>
<td>200</td>
<td>9.80</td>
<td>15.62</td>
<td>200</td>
<td>5.53</td>
<td>19.89</td>
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<tr>
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<td>11.38</td>
<td>21.19</td>
<td>250</td>
<td>8.09</td>
<td>24.48</td>
</tr>
<tr>
<td>300</td>
<td>12.84</td>
<td>26.88</td>
<td>300</td>
<td>10.25</td>
<td>29.47</td>
</tr>
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</table>

### Table 4.6b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Volumes of Transfused Red Blood Cells per Kilogram Body Weight at Biopsy in SCD

<table>
<thead>
<tr>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
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<td>1.13</td>
<td>9.99</td>
</tr>
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<td>8.14</td>
<td>10.13</td>
<td>150</td>
<td>4.87</td>
<td>13.40</td>
</tr>
<tr>
<td>200</td>
<td>10.87</td>
<td>14.54</td>
<td>200</td>
<td>8.17</td>
<td>17.24</td>
</tr>
<tr>
<td>250</td>
<td>13.19</td>
<td>19.38</td>
<td>250</td>
<td>11.11</td>
<td>21.46</td>
</tr>
<tr>
<td>300</td>
<td>15.42</td>
<td>24.29</td>
<td>300</td>
<td>13.79</td>
<td>25.93</td>
</tr>
</tbody>
</table>

### Table 4.7a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Volumes of Transfused Red Blood Cells per Kilogram Body Weight at Biopsy in TM

<table>
<thead>
<tr>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
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<td>0</td>
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</tr>
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<td>150</td>
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<td>150</td>
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<td>15.17</td>
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<td>7.87</td>
<td>20.98</td>
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<tr>
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<td>20.06</td>
<td>300</td>
<td>10.41</td>
<td>24.08</td>
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### Table 4.7b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Volumes of Transfused Red Blood Cells per Kilogram Body Weight at Biopsy in TM

<table>
<thead>
<tr>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
<th>RBC Transfused (cc/kg)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
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<td>19.03</td>
<td>300</td>
<td>12.89</td>
<td>21.59</td>
</tr>
<tr>
<td>SFC (ng/mL)</td>
<td>HIC Lower limit mgFe/g liver, dry weight</td>
<td>HIC Upper limit mgFe/g liver, dry weight</td>
<td>SFC (ng/mL)</td>
<td>HIC Lower limit mgFe/g liver, dry weight</td>
<td>HIC Upper limit mgFe/g liver, dry weight</td>
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<td>------------</td>
<td>----------------------------------------</td>
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<td>5000</td>
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<td>23.73</td>
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</table>

<table>
<thead>
<tr>
<th>SFC (ng/mL)</th>
<th>HIC Lower limit mgFe/g liver, dry weight</th>
<th>HIC Upper limit mgFe/g liver, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>5.34</td>
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<tr>
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<td>10.19</td>
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<td>9.94</td>
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<td>2000</td>
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<td>9.98</td>
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<tr>
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</table>

Table 4.8a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Serum Ferritin Concentrations (SFC) in Sickle Cell Disease

Table 4.8b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) & for an Individual (right) at Selected Serum Ferritin Concentrations (SFC) in Sickle Cell Disease

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### Table 4.9a. 95% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Serum Ferritin Concentrations in Thalassemia major

<table>
<thead>
<tr>
<th>SFC (ng/mL)</th>
<th>HIC Lower limit (mgFe/g liver, dry weight)</th>
<th>HIC Upper limit (mgFe/g liver, dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>3.47</td>
<td>9.05</td>
</tr>
<tr>
<td>1000</td>
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<td>11.57</td>
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<tr>
<td>3500</td>
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<td>21.40</td>
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<td>4000</td>
<td>12.88</td>
<td>24.15</td>
</tr>
<tr>
<td>4500</td>
<td>13.61</td>
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<tr>
<td>6000</td>
<td>15.74</td>
<td>35.29</td>
</tr>
</tbody>
</table>

### Table 4.9b. 80% Prediction Intervals for the Mean Hepatic Iron Concentration (left) and for an Individual (right) at Selected Serum Ferritin Concentrations in Thalassemia major

<table>
<thead>
<tr>
<th>SFC (ng/mL)</th>
<th>HIC Lower limit (mgFe/g liver, dry weight)</th>
<th>HIC Upper limit (mgFe/g liver, dry weight)</th>
</tr>
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<tbody>
<tr>
<td>500</td>
<td>4.49</td>
<td>8.04</td>
</tr>
<tr>
<td>1000</td>
<td>6.68</td>
<td>9.35</td>
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<tr>
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<td>8.62</td>
<td>10.91</td>
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<td>12.85</td>
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<td>17.35</td>
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<tr>
<td>3500</td>
<td>13.82</td>
<td>19.71</td>
</tr>
<tr>
<td>4000</td>
<td>14.93</td>
<td>22.10</td>
</tr>
<tr>
<td>4500</td>
<td>16.03</td>
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<tr>
<th>SFC (ng/mL)</th>
<th>HIC Lower limit (mgFe/g liver, dry weight)</th>
<th>HIC Upper limit (mgFe/g liver, dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0.60</td>
<td>11.93</td>
</tr>
<tr>
<td>1000</td>
<td>2.47</td>
<td>13.55</td>
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<tr>
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<tr>
<td>6000</td>
<td>17.30</td>
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Figure 4.4. The correlation between the Total Iron Score from microscopic hepatic tissue analysis and the Quantitative Biochemical Hepatic Iron Concentration in Sickle Cell Disease (a), and in Thalassemia (b).
Figure 4.5. Distribution of hepatic iron in transfused patients with sickle cell disease (red) and thalassemia (blue), (a) reporting the mean iron score within the liver and its various iron compartments and expressed as a percentage of the total iron identified in the liver, (b) reporting the distribution of iron relative to the maximum capacity of each storage compartment. Error bars represent standard deviations.

In regularly transfused patients with sickle cell disease and thalassemia, most of the iron that accumulates in the liver is stored within hepatocytes. Comparing the SCD and TM group, the percentage of total liver iron accounted for by hepatocytes (72.7% vs 67.2%, P=0.11), Kupffer cells (22.2% vs 25.1%, P=0.42), and portal tracts (5.1% vs 7.7%, P=0.14%), is shown.
4.4.4 Fibrosis and Portal Inflammation

In the SCD group, liver damage was detected in 6 patients (33.3%) with a mean liver iron of $9.9 \pm 0.5 \text{ mg/g}$, dry weight (range, 6.2 to 14.6) (Figure 4.6). Three patients had evidence of both liver fibrosis and portal inflammation. Liver fibrosis alone was also detected in two patients, and portal inflammation alone was found in one other patient. In the TM group, liver damage was detected in 10 patients (58.8% of 17 evaluable biopsies) with a mean liver iron of $9.9 \pm 1.8 \text{ mg/g}$, dry weight (range, 2.8 to 20.1) (Figure 4.6). Three patients had evidence of both liver fibrosis and portal inflammation. Liver fibrosis alone was also detected in three patients, and portal inflammation alone was found in four other patients. No significant differences in the incidence of liver damage were observed between the two groups. All patients were negative for Hepatitis C viral infection.
Figure 4.6. Incidence of liver damage in transfused patients with Sickle Cell Disease (red) and Thalassemia (blue). Hepatic fibrosis (a) and/or portal inflammation (b) was detected in patients with a range of HICs, notably even below 7mg/gdw, but was also absent in patients with a range of HICs reaching as high as 16.9 mg/gdw.
4.5 Discussion

Initial reports characterizing thalassemia described increased body iron levels in children who were transfused irregularly and died before their eighth birthday. Hepatic iron concentrations ranged between 3.8 to 13.2 mg/g, dw (Whipple and Bradford, 1936). Liver fibrosis associated with extensive hepatic siderosis and the accumulation of iron in various other organs, was also identified histologically in another group of 12 transfused thalassemia patients, 5 of whom died at ages between 4 to 27 years, and all with fibrosis within the first 6 years of life (Ellis et al., 1954). Witzleben and Wyatt (1961) also reported 4 deaths before the age of 13 years, in transfused thalassemia patients. All had final hepatic iron concentrations exceeding 25mg/g,dw, and all developed cirrhosis and heart disease, including an additional patient still alive at 16 years of age. Risdon et al (1975) observed an incidence and progression of fibrosis among thalassemia patients, which was accelerated at elevated hepatic iron concentrations. Liver disease and fibrosis was also identified in a group of 26 regularly transfused patients with thalassemia under 14 years of age (Masera et al., 1976). A tremendous breakthrough in early 1960s, was the development of deferoxamine, and several studies focussing on iron accumulation and progression of disease in thalassemia, have been confounded by the use of deferoxamine chelation therapy. Maurer et al (1988) reported pre-chelation hepatic iron concentrations ranging from approximately 18mg/g,dw to over 60mg/g,dw, in a group of 16 regularly transfused thalassemia patients between 3 to 17 years of age, and identified a correlation of r=0.82 between the HIC and the number of transfusions pre-chelation. However, the paper does not provide enough data to suggest a pre-chelation rate of transfusional iron accumulation. Therefore, clinicians treating thalassemia are continuing to struggle with the management of iron overload, and have been searching for ideal indicators of body iron levels to monitor iron accumulation.

Frumin and Miller (1952) were the first to report exogenous hemochromatosis in transfused sickle cell disease, as they identified a striking amount of iron deposits within the liver of a four-year old sickle cell patient who had received 18 liters of transfused blood over 3 years. Green et al. (1953) also suggested that frequent blood transfusions cause hepatic dysfunction in sickle cell disease, as a result of the deposition of iron
contained within transfused blood. Bogoch et al (1955) also acknowledged that hepatic fibrosis and cirrhosis could possibly be related to increased amounts of hepatic iron as a result of multiple blood transfusions. Along with hepatic abnormalities, endocrine and cardiac disease, associated with increased body iron stores, have also been reported in sickle cell disease (Buja and Roberts, 1971; Cohen et al., 1984 abstract). Finch et al (1982) also identified hepatic fibrosis and heavy deposits of hepatic iron in a patient with sickle cell disease who had received more than 100 transfusions over an 11-year period prior to biopsy, and a progression to cirrhosis, increased iron loading, and liver dysfunction after an additional 13 years of regular transfusion therapy. However, the authors concluded that the massive iron overload, also detected in the heart and various endocrine organs, produced no clinical symptoms other than osteoporosis, and the patients’ death at age 47 years remained unexplained. Several studies have since verified transfusional iron overload in sickle cell disease using various indirect and non-quantitative indicators of body iron levels, but could not accurately quantify iron accumulation nor have they identified any correlation between body iron burden and transfusional iron load (Washington and Boggs, 1975; Hussain et al., 1978; Buffone et al., 1980).

An extensive review of the literature reveals that there is currently no conclusive evidence to suggest that regularly transfused patients with sickle cell disease accumulate and store iron at different rates or through different mechanisms than patients with thalassemia. In addition, the actual rates at which these two groups of regularly transfused patients accumulate iron has not been accurately quantified and compared. Attempts to quantify these rates have used patients already receiving chelation therapy, or have used inadequate techniques to accurately quantify body iron levels. Therefore, this is the first study using reliable quantitative measures of body iron concentration to quantify the rates of iron accumulation in regularly transfused patients with sickle cell disease and thalassemia before being introduced to any chelation therapy.

Patients with severe thalassemia syndromes require life-sustaining regular red blood cell transfusions, initiated at an early age to maximize the potential for normal growth and development, while the clinical indications for regular transfusion in sickle cell disease are usually manifested later in the first or earlier in the second decade of life.
This difference was evident between our patients with sickle cell disease and thalassemia. However, the volumes of blood (RBCs) transfused per kilogram body weight, and the frequency of transfusions, in both groups were similar, despite the difference in transfusion strategies (ie. thalassemia strategy to maintain baseline hemoglobin levels above 9.5 g/dl; SCD strategy to maintain HbS levels below 30% of total hemoglobin). Therefore, in the absence of chelation therapy, we hypothesized that similar rates of transfusion would result in similar rates of iron accumulation into body iron stores.

**Duration of Transfusion Therapy**

In both sickle cell disease and thalassemia patients, initial hepatic iron concentration correlated reasonably well with the number of months of transfusion therapy, and similar rates of iron accumulation between the two groups are apparent (Fig 4.1). Thus, monitoring the number of months of transfusion therapy appears to be a useful gauge of iron burden in both thalassemia and sickle cell disease. From other studies in sickle cell disease, significant correlation between HIC and the duration of transfusion therapy have also been recently reported ($r=0.795$ and $r=0.60$), although several patients in each series were already receiving chelation therapy (Harmatz et al., 2000; Vieira et al., 1999). From our series, it appears that approximately 13 months following the initiation of regular transfusion therapy, a majority of patients in both groups have already achieved body iron levels that exceed the previously described 7mg/g,(dw threshold for increased risk of iron-induced complications (Niedereau et al., 1996; Olivieri, 1999).

Using linear regression analyses, we can more accurately conclude with 95% confidence, that sickle cell patients have an average HIC above 7mg/g, dw after 15 months of transfusion therapy. Furthermore, we can predict with 95% confidence that after 20 months of transfusion therapy, every individual SCD patient will have an HIC above 5.2mg/g, dw and as high as 18.6mg/g, dw. Broadening our confidence to 80%, we can predict that after 15 months of transfusions, every sickle cell patient will have an HIC above 4.4mg/g, dw and as high as 12.6mg/g, dw (Tables 4.4a,b).
Similarly, we can conclude with greater than 95% confidence that regularly transfused thalassemia patients also have an average HIC above 7mg/g,dw after 15 months of transfusions, and can predict with 95% confidence that after 20 months of transfusion therapy, every individual thalassemia patient will have an HIC above 4.1mg/g,dw and as high as 18.3mg/g,dw. Broadening our confidence to 80%, we can predict that after 15 months of transfusions, all thalassemia patients will have an HIC above 4.3mg/g,dw and as high as 13.3mg/g,dw (Tables 4.5a,b).

**Transfusional Volume**

The total volume of transfused red cells per kilogram body weight also appears to be a useful measure of iron accumulation, revealing a moderate correlation with hepatic iron concentration in both thalassemia and sickle cell disease (Figure 4.2). Similar rates of iron accumulation among thalassemia and sickle cell patients is again illustrated. Vieira et al (1999) also reported a similar correlation of r=0.53 between HIC and the total volume of transfused blood per kilogram body weight measured at biopsy. In our series, both sickle cell and thalassemia patients appear to be reaching the hepatic iron 7 mg/g,dw threshold of increased risk after approximately 110 - 130 mL of RBCs transfused per kilogram body weight at biopsy. Furthermore, HIC exceeds the 15 mg/g,dw threshold of increased risk of cardiac disease and early death after approximately 230 - 250 mL of RBCs transfused per kilogram body weight at biopsy.

Confidence intervals indicate with 95% confidence that after 150 cc/kg of transfused red cells, the mean HIC in both groups exceeded 7 mg/g,dw. Once again, we can predict with 95% confidence that after 150 cc/kg, all sickle cell disease and thalassemia patients will have an HIC above 2.4mg/g,dw, and with 80% confidence that their HIC exceeds 4.7 mg/g,dw (Tables 4.6a,b and 4.7a,b).

**Liver Damage and Liver Iron Distribution**

Liver disease secondary to iron overload has been recognized as a common cause of death in young adults with β-thalassemia (Zurlo et al., 1989). In 1977, Iancu et al
(1977) reported a high incidence of hepatic fibrosis in children with β-thalassemia under 3 years of age. Therefore, it appears that iron quickly accumulates in the liver within the initial years of transfusion therapy causing fibrosis, which eventually may progress to cirrhosis. Hepatic damage was identified in several of our patients, almost all with liver irons approaching or already exceeding the 7 mg/g,dw threshold of increased risk. Furthermore, the proportion of our patients in whom hepatic damage (fibrosis and/or portal inflammation) was identified, was similar in patients with sickle cell disease and thalassemia. However, recent studies have reported that iron overloaded patients with sickle cell disease do not appear to develop several of the iron-related complications common among iron overloaded thalassemia patients, suggesting that SCD patients may be able to tolerate elevated iron levels through a protective mechanism (Finch et al., 1982; Harmatz, 2000). However, confounding influences among the patients studied by Harmatz included (1) 91% (70/77) of patients were already receiving DFO chelation therapy, capable of arresting the progression of hepatic fibrosis, even at massively elevated hepatic iron concentrations (Barry et al., 1974), and (2) patients with SCD were transfused and exposed to elevated iron levels for a shorter period of time (BTM group transfused for a mean of 13.6 years, while SCD group transfused for a mean of 6.0 years), and perhaps were assessed before iron-related complications may have had a chance to develop (Harmatz et al., 2000). Nevertheless, the authors suggest a difference in iron storage between the two disorders, favoring RE cell iron storage in sickle cell disease, where the iron is believed to be relatively harmless (Hershko and Weatherall, 1988). However, the results of our study can at least confirm that after approximately 20 months of regular transfusions, there was no difference in liver iron distribution observed between the SCD and TM groups, and similar proportions of patients in both groups developed liver fibrosis. Therefore, a protective mechanism seems unlikely, or becomes manifested later in life, or perhaps as iron concentrations continue to rise. Currently there is no evidence to suggest that iron accumulation and distribution would not continue to be similar between the two groups of patients.

Turlin and Deugnier (1998) observed very different patterns of iron distribution in the livers of patients with transfusional iron overload compared to those with iron overload due to hyperabsorption (characteristic of patients with hemochromatosis). In
transfused patients, hepatic iron appeared to be deposited primarily in Kupffer cells and scarcely in parenchymal stores, while the opposite was noted in hyperabsorptive disorders. Harmatz et al (2000) confirmed the observations of Turlin and Deugnier, after observing a greater relative deposition of iron within Kupffer cells, in regularly transfused, heavily iron overloaded sickle cell patients (50% with HIC > 15mg/gdw).

Our results of liver iron distribution present a different picture of iron accumulation within hepatocytes and Kupffer cells, however our patients had only been transfused for a mean of approximately 20 months, and were not as heavily iron overloaded. We observed no difference in relative iron deposition between hepatocytes and Kupffer cells in both groups of patients. (The greater absolute parenchymal iron score compared to the Kupffer cell iron score, despite similar iron saturation within each compartment, is reflective of the approximately three-fold greater number of hepatocytes than Kupffer cells in the liver). Furthermore, we identified a clear periportal-to-centrilobular iron concentration gradient among hepatocytes, previously believed to be atypical of transfusional iron overload (Turlin and Deugnier, 1998). Combining our results with the results of Harmatz et al (2000), it appears that iron distribution is altered once transferrin becomes completely saturated, and/or in response to chelation therapy.

Our observations suggest that during the initial years of transfusional iron accumulation, there appears to be no selective deposition and storage of iron within either parenchymal or mesenchymal (Kupffer cell) stores. The overflow from Kupffer cell to hepatocyte mechanism suggested by Harmatz (2000), would not appropriately explain the early distribution of hepatic iron observed in our SCD and TM patients.

Our observations on iron distribution agree with the observations and mechanism suggested by Schafer et al (1981), Cazzola and Barosi (1982), and Cazzola et al (1983), that initially transfusional iron is not primarily stored in the reticulo-endothelial system, and is instead rapidly redistributed to parenchymal tissue. This emphasizes how the distribution of storage iron is influenced by transferrin saturation, which over the first year of transfusion therapy, consistently increases and approaches 100% in response to the accumulation of transfusional iron (Modell and Berdoukas, 1984). While transferrin remains incompletely saturated, it can accommodate the iron that is processed through the reticulo-endothelial cell system. However, as the transferrin saturation approaches
100%, RE cells that continue to process old and defective erythrocytes, may be less likely to release iron and may alternatively store it. At this point, RE cell iron accumulation may be augmented and relative iron distribution may begin to favor RE cells.

**Serum Ferritin Concentration**

Currently, most clinicians base the decision to begin chelation therapy in regularly transfused patients, on measures of the serum ferritin concentration after a period of regular transfusions. The serum ferritin concentration remains the most common and inappropriately reported parameter for measuring and monitoring body iron levels. High levels of storage iron do influence the serum ferritin concentration, however other influences include ascorbate deficiency, fever, infection, inflammation, hepatic damage, hemolysis, and ineffective erythropoiesis (Olivieri and Brittenham, 1997). Several reports have revealed that serum ferritin levels do not quantitatively reflect body iron levels, and are thus limited in their clinical usefulness (Walters et al., 1973; Birgegard et al., 1977; Jacob et al., 1980; De Virgillis et al., 1980; Worwood et al., 1980). Although undisputedly less invasive than liver biopsies, recently reported correlations between SFC and HIC in patients with thalassemia, ranged between 0.69 to 0.76 (Brittenham et al., 1993; Nielsen et al., 1995; Piga et al., 1999). Even the strongest of these correlations (r=0.76: Brittenham et al., 1993), produces 95% confidence intervals which are far too broad to be of any clinical use, especially regarding chelation therapy. The moderate correlation observed in our thalassemia group (r=0.61) was similar to the correlation observed by these other groups. Earlier studies must also be acknowledged, which reported HIC vs SFC correlations ranging between r=0.159 to r=0.96 (De Virgillis et al., 1980; Prieto et al., 1975; Aldouri et al., 1987). In children with thalassemia, De Virgillis et al. (1980) also reported no correlation between serum ferritin concentration and the numbers of transfusions (r=0.176, p-value reported as not significant). Worwood et al (1980) reported a correlation between SFC and the volume of blood transfused of only r=0.38 among regularly transfused thalassemia major patients.

Although Brittenham et al. (1993) also reported a correlation of r=0.75 between SFC and HIC in Sickle Cell Disease, several other investigators have reported much
weaker and often insignificant correlations, ranging between $r=0.04$ to $r=0.35$ (Vieira et al., 1999; Harmatz et al., 2000). These groups, along with others, have also reported very weak correlation between SFC and both the duration of regular transfusion therapy ($r=0.308$: Harmatz et al., 2000) and the total volume of blood transfused ($r=0.05$: Vieira et al., 1999; Hussain et al., 1978; Adekile et al., 1985). Altogether, these observations strongly suggest that serum ferritin may be an inaccurate and inappropriate marker for iron overload in sickle cell disease. Based on the correlation reported in the literature between SFC and HIC in patients with sickle cell disease, we expected to observe a similar discouraging correlation in our patients. The correlation observed in our SCD group was much worse than the correlation in our thalassemia group, and statistically no correlation exists ($p=0.669$), agreeing with previous the reports (Vieira et al., 1999; Harmatz et al., 2000).

**False negative and false positive**

Olivieri et al (1994) identified an improved prognosis of survival without cardiac disease in regularly transfused BTM patients maintaining serum ferritin concentrations below 2500 ng/ml. Although clearly not designed to establish a clinical SFC threshold to guide chelation therapy, many clinicians may acknowledge this interpretation. Emphasizing how inappropriately this threshold would have guided clinicians, the initiation of chelation may have been delayed in approximately 78% (7/9) of patients with sickle cell disease, and 88% (14/16) of patients with thalassemia, who had body iron levels exceeding the 7mg/g,dw threshold of increased risk of iron-related complications.

Recently, two reports have suggested that complications can be avoided when the SFC is maintained below 1500ng/mL, and indicating when to begin chelation therapy (Telfer et al., 2000; Ballas, 2001). Based on these suggestions, the decisions on chelation therapy may have been inappropriately delayed in approximately 44% (4/9) of individuals with sickle cell disease and 69% (11/16) with thalassemia, who actually had body iron levels already within the range of increased risk of complications (>7mg/g,dw). Including the number of patients who may have been prematurely introduced to chelation therapy.
therapy, approximately 67% and 64% of serum ferritin concentrations may have misguided treatment in our sickle cell disease and thalassemia patients, respectively.

Altogether, these observations strongly suggest that serum ferritin is an inaccurate and inappropriate marker for iron overload in both sickle cell disease and thalassemia. We suggest that clinicians should not be considering the serum ferritin concentration when making any decisions about when to begin chelation therapy.

4.5.1 Conclusion

Patients with sickle cell disease and thalassemia introduced to regular transfusions, experience similar rates of iron accumulation over the first two years of transfusion therapy (prior to chelation). Furthermore, there appears to be no difference in the distribution of iron into parenchymal (hepatocytes) and reticuloendothelial (Kupffer cell) iron stores between the two groups.

At the present time, chelation therapy is started at arbitrary time points after beginning regular transfusion therapy, due to the lack of precision of markers or indicators of body iron. These data suggest that the use of the serum ferritin concentration to initiate chelation therapy in regularly transfused patients with sickle cell disease or thalassemia, may mislead clinicians anxious to avoid iron-induced toxicity in their patients. Therefore, serum ferritin should not be used as a guide to initiate chelation therapy. Since a substantial proportion of patients already have elevated liver irons and evidence of hepatocellular damage (which is arrested with chelation therapy using desferrioxamine) one to two years following the initiation of regular transfusions, our recommendation is that hepatic iron and histology be evaluated in all patients with thalassemia and sickle cell disease approximately 12 months following the initiation of regular transfusions.
4.5.2 Future Direction

Recent studies have suggested that patients with sickle cell disease are not as susceptible to iron-induced organ damage as are patients with thalassemia; the proposed increased tolerance to iron related to an augmented reticulo-endothelial system characteristic of patients with sickle cell disease (Harmatz et al., 2000). This proposition is being challenged by our research group, as the reliability of these studies are limited by the fact that (1) thalassemia patients had been regularly transfused for longer intervals than sickle cell patients, exposing them to elevated iron levels for a significantly longer period of time, and (2) 91% (70/77) of patients were receiving deferoxamine chelation, protecting against iron toxicity. A more reliable study would prospectively compare the incidence or development of secondary complications of iron overload among sickle cell and thalassemia patients exposed to elevated body iron levels for similar periods of time.

Another important study could focus on identifying the length of time or duration of exposure to elevated body iron concentrations required to develop the complications associated with iron-overload.

Once transferrin becomes fully saturated after a period of transfusions, do RE cells adjust the proportion of iron that they release (normally to transferrin) and the iron that they store, as they continue to process old and defective red cells. By what mechanism might iron release from these RE cells be suppressed, and a greater proportion of iron transferred to these cell stores?

Another related topic of interest also focuses on transferrin saturation, mechanisms of RE cell iron processing and storage, and non-transferrin bound plasma iron (NTBPI), probably the most toxic form of circulating and storage iron, found in iron-overloaded patients. Several groups have already begun studying the role of NTBPI in iron-induced organ damage, while others are developing, and determining the clinical usefulness of, serological assays to detect when NTBPI initially appears in the plasma, and it's potential as an indicator of iron overload.
Chapter 5

EVALUATION OF THE IRON CHELATION EFFICIENCY OF DEFEROXAMINE (DFO) AND DEFERIPRONE (L1) IN THALASSEMAIA MAJOR WITH BALANCE STUDIES USING HEPATIC IRON CONCENTRATION (HIC) TO DETERMINE BODY IRON STORES

5.1 Background and Literature Review

Patients with severe thalassemia syndromes require life-preserving regular transfusion therapy, initiated at an early age to sustain normal growth and development throughout the first decade of life. A moderate transfusion regimen maintaining baseline pre-transfusion hemoglobin between 9–10 g/dl provides adequate relief of anemic symptoms, suppresses endogenous erythropoiesis, and inhibits gastrointestinal iron absorption (Cazzola et al., 1997; Olivieri, 1999). Based on a transfused red cell survival of 90 days, this is achieved through blood transfusions every four weeks using a transfusional volume of 10.5 cc of red blood cells per kilogram body weight. However, without iron chelation therapy, these patients rarely survive beyond the second or third decade of life, as transfusional iron accumulates within body iron stores, notably the liver, heart, and endocrine tissues, and patients eventually succumb to heart disease associated with iron toxicity.

In the 1960s, the greatest breakthrough in the treatment of thalassemia since regular transfusion therapy, was the discovery of deferoxamine, a trihydroxamic acid derived from a naturally occurring siderophore (iron-binding protein) produced by the fungus Streptomyces pilosus (Keberle, 1964). DFO is a hexadentate iron chelator, capable of binding to the six coordination sites of ferric iron in a 1:1 iron:DFO complex (feroxamine), trapping iron in a metabolically inactive form, which is excreted in both the urine and feces (Pippard et al., 1982). Routine treatment of iron overload using DFO chelation therapy began in the 1970s, and several studies have since reported and
confirmed that adequate DFO therapy, preventing the accumulation of iron to toxic levels, can:

1. Prevent cardiac disease and early death (Brittenham et al., 1994).
2. Arrest the progression of hepatic fibrosis, and development of cirrhosis (Barry et al., 1974).
3. Prevent various endocrine disorders, including pancreatic, thyroid, parathyroid, adrenal abnormalities, and abnormal sexual maturation (Bronspiegel-Weintrob et al., 1990)

Presently, deferoxamine remains the only iron chelator approved for clinical use in the treatment of transfusional iron overload. However, the two major disadvantages of DFO, (1) it must be administered through prolonged parenteral infusions, as it is poorly absorbed orally due to its bulky size and rapidly metabolized in the plasma (Callendar and Weatherall, 1980; Summers et al., 1979), and (2) the cost of both the drug and the equipment for its administration, have prompted the search for a less expensive and orally active iron chelator.

The most extensively studied oral iron chelator has been 1,2-dimethyl-3-hydroxypyridin-4-one, also known as deferiprone, or L1. Deferiprone is a neutral, bidentate iron chelator, which forms a 1:3 ferric iron:L1 complex (3 molecules of L1 engulf 1 ferric iron molecule, occupying its 6 coordination sites), which is excreted mainly in the urine (Olivieri and Brittenham, 1997). Deferiprone was initially reported to have beneficial short-term effects on iron balance, and was also associated with an improvement in patient compliance with chelation therapy (Olivieri et al., 1995). However, long-term studies identified an eventual stabilization or increase in hepatic iron concentration in deferiprone-chelated patients, as well as other possible deleterious effects including the progression of hepatic fibrosis, neutropenia, and agranulocytosis (Hoffbrand et al., 1998; Olivieri et al., 1998).

Currently, iron chelation therapy is initiated and adjusted based on either hepatic iron concentrations, or more commonly serum ferritin concentrations, after a period of transfusion. Standard, routine doses of 25 mg/kg/day for 5 nights/week are usually
initiated approximately 1 year after starting transfusions, or when the HIC exceeds 3.2 mg/g, dry weight (Olivieri and Brittenham, 1997). The dose is then adjusted based on fluctuations in HIC, serum ferritin trends, or inadequate compliance with therapy. Prescriptions are not individualized, as clinicians are unable to accurately and quantitatively predict iron balance in regularly transfused patients receiving chelation therapy. Although we can anticipate the amount of transfusional iron a patient will receive, we cannot predict how much iron will be removed through chelation therapy, as we do not know how efficiently iron chelators remove iron from the body.

Angelucci et al. (2000) have recently identified a direct and reliable relationship between hepatic iron concentration and the concentration of the total body iron store (TBIS). Therefore, it is now possible to calculate net iron balance using serial hepatic iron concentrations measured biochemically using liver biopsy tissue weighing greater than 1.0mg, dry weight (Angelucci et al., 2000). Magnetic quantitation of hepatic iron concentration using a SQUID susceptometer has been proven to be as accurate and reliable as biochemical iron quantitation, and therefore can also be used for iron balance calculations (Brittenham et al., 1982, 1988, 1993; Fischer et al., 1999).

Chelation efficiency is defined as the amount of iron that 1 mole of a chelator is actually able to remove, compared to the amount of iron that the chelator is theoretically able to remove. In theory, one mole of DFO or 3 moles of L1 are able to remove 1 mole of iron. Currently, the chelation efficiencies of DFO and L1 are not known. This is the first study proposing to be able to accurately calculate the efficiency of iron chelation therapy using measures of iron balance, combined with the amounts of transfusional iron and chelation administered to a patient over an interval between HIC measures. Clinicians may be able to use this chelation efficiency to finally quantitatively determine the dose of chelation required to achieve negative iron balance, which would reduce body iron levels in patients with, or at risk of, iron overload.

The purpose of this study was to determine the chelation efficiency of DFO and L1 in patients with β-Thalassemia major, and define new algorithms to determine the dose of chelation required to maintain or achieve optimal body iron levels. These algorithms calculate individualized prescriptions of chelation therapy for iron overloaded patients with β-Thalassemia major.

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5.2 Experimental Design

5.2.1 Sample Population

The charts of all β-Thalassemia major patients in our paediatric and adult hemoglobinopathy programs were reviewed, identifying regularly transfused patients who had serial SQUIDs and/or liver biopsies for direct quantitative magnetic or biochemical measure of hepatic iron concentration, while on chelation therapy with either deferoxamine (DFO) or deferiprone (L1).

5.2.1.1 Inclusion/Exclusion Criteria

All patients included in this study were non-cirrhotic. HIC intervals, defined as the period of time between consecutive biopsies or SQUIDs, were excluded when the initial or final HIC was quantified using a sample of biopsy tissue weighing less than 1.0 mg (dry weight), a recently reported threshold for optimal accuracy in biochemical hepatic iron quantitation (Angelucci, 2000). Furthermore, only patients with complete blood transfusion records (dates, body weights, transfusion volumes, hematocrits) and complete chelation records (dates, body weights, doses, compliance records) throughout the HIC interval were studied.

5.2.1.1.1 DFO Chelation and CADD-Pump Compliance

All DFO-chelated patients included in this study received subcutaneous infusions of DFO dissolved in saline, administered through a Continuous Ambulatory Drug Delivery, or CADD-pump, equipped with an electronic volume counter for compliance monitoring. These pumps automatically count every milliliter of the DFO-solution that is actually infused into the patient. Ideally, DFO-chelated patients should have their CADD-pumps readings recorded every month, conveniently on the days of their regular transfusions. For this study, compliance records containing gaps of greater than 3 months within an HIC interval, were considered inaccurate and unreliable, and the interval would be excluded. Furthermore, an interval was excluded whenever compliance was not
recorded on a day when the dose of DFO was changed, making it impossible to accurately determine how much DFO the patient actually received before and after the dose was changed. Finally, an interval was also excluded whenever a pump malfunctioned, as we couldn’t determine the amount of DFO the patient received over the days since the last reading before the pump malfunctioned.

5.2.1.1.2 L1 Chelation and MEMS-Cap Compliance

L1 compliance was monitored using both, pill counts, and the Medication Event Monitoring System (MEMS, Aprex Corporation), a medication bottle cap equipped with a microprocessor that records every date and time the L1 bottle is opened. Ideally, at every clinic visit, patients returned the bottle and cap, the number of pills remaining was recorded, and the MEMS cap was connected to a computer to download the interval compliance information. L1 patients were included in this study if compliance records were complete, accounting for every day of therapy, and only if their compliance from pill counts matched the compliance from the MEMS.

5.2.1.2 Measures of Hepatic Iron Concentration

Direct biochemical measurement of hepatic iron concentration, or HIC, using a sample of liver tissue provides the most quantitative assessment of body iron burden (Pippard, 1989). Magnetic susceptometry of the liver using a Superconducting Quantum Interference Device (SQUID) is currently the only other technique capable of measuring hepatic iron concentration with comparable accuracy and reliability (Brittenham et al., 1982, 1988, 1993). Between July 1993 and December 1999, 35 regularly transfused beta-thalassemia major patients were either sent for SQUID hepatic iron quantitation or had a percutaneous liver biopsy to acquire tissue for biochemical iron quantitation. All tissue samples analyzed, weighed greater than 1.0mg, dry weight, a recently reported threshold for optimal accuracy (Angelucci et al., 2000). Hepatic iron concentrations are reported in milligrams of iron per gram of liver tissue, dry weight (mg/gdw). All 35 patients were receiving regular chelation therapy with either subcutaneous deferoxamine (n=12;
6M,6F) or oral deferiprone (n=23;11M,12F). The 12 patients in the DFO group provided 16 evaluable intervals, while the 23 L1 patients provided 31 evaluable intervals. Patient characteristics are reported in Table 5.1.

<table>
<thead>
<tr>
<th>Table 5.1. Characteristics of 35 regularly-transfused thalassemia patients with adequate intervals for calculating chelation efficiency.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Patients (M/F)</strong></td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>DFO</td>
</tr>
<tr>
<td>L1</td>
</tr>
<tr>
<td><strong>Age at Start of Interval (years)</strong></td>
</tr>
<tr>
<td>DFO</td>
</tr>
<tr>
<td>L1</td>
</tr>
<tr>
<td><strong>Weight at Start of Interval (kg)</strong></td>
</tr>
<tr>
<td>DFO</td>
</tr>
<tr>
<td>L1</td>
</tr>
<tr>
<td><strong>HIC at Start of Interval (mg/g, dry weight)</strong></td>
</tr>
<tr>
<td>DFO</td>
</tr>
<tr>
<td>L1</td>
</tr>
</tbody>
</table>

5.2.1.3 Transfusion Records

All patients received regular red blood cell transfusion therapy. The goal of transfusion therapy was to maintain the hemoglobin concentration above 9.5 g/dL. DFO-chelated patients were transfused 14.6 ± 0.3 (range, 10.9 - 16.4) mL of blood per kg body weight, every 28.0 ± 0.7 (range, 24.1 - 35.6) days. L1-chelated patients were transfused similar volumes of blood (per kilogram body weight) 14.2 ± 0.3 (range, 11.1 - 16.6) mL/kg (p=0.30), however the period between transfusions was slightly longer, every 30.0 ± 0.6 (range, 26.7 - 44.0) days (p=0.04). Transfusion records indicated the volume of blood transfused between serial HIC measures, which was multiplied by the hematocrit of transfused blood in our program (0.7), to calculate the total volume of red blood cells transfused over the interval. Based on the assumption that 1.0mL of red blood cells contains 1.0 mg of iron (Reed et al., 1999), this also identifies the amount of transfusional iron received over the interval.
5.2.1.4 Chelation Records

DFO was prescribed at a mean daily dose of 38.2 ± 3.4 (range, 10.9 – 65.2) mg/kg/day, dissolved in saline and infused 7 nights/week. However, the concentration of the DFO solution and/or the volume infused, could be adjusted to retain the same dosage while infusing for fewer nights per week. All DFO-chelated patients also received ascorbic acid (vitamin C) supplementation, 100 mg, administered 30 – 60 minutes after the start of every DFO infusion. L1 was prescribed at a mean daily dose of 74.3 ± 0.7 (range, 63.5 - 86.8) mg/kg/day. The toxicity of ascorbate supplementation during L1 therapy is unknown, and L1 patients did not receive supplementation. From chelation and compliance records we were able to calculate the total milligrams of chelation received over an interval between HICs, which could then be converted into moles based on molecular weights of 656.8 g/mol for deferoxamine and 139.2 g/mol for deferiprone.

5.2.1.5 Conversion of Hepatic Iron Concentration into the Total Body Iron Store

Hepatic iron concentration (HIC), reported in milligrams of iron per gram of liver tissue, dry weight (mg/gdw), was converted to an estimate of the total amount of iron stored in the body, referred to as the total body iron store (TBIS), reported in millimoles of iron. This conversion is based on the equation derived by Angelucci et al. (2000), who quantified both the change in mobilizable storage iron eliminated through regular phlebotomy therapy, and the corresponding change in hepatic iron concentration, which correlated almost perfectly with each other when biopsy tissue samples weighed greater than 1.0mg, dry weight.

\[
[\text{TBIS}]_{(\text{mmol})} = 10.6_{(\text{g/kg})} \times \text{HIC}_{(\text{mg/g,dw})} \quad \text{(Angelucci et al., 2000)}
\]

Factoring in the patient’s weight and the molecular weight of iron (55.85g/mol) provides the estimate of the total number of moles of iron within the total body iron store.

\[
\text{TBIS}_{(\text{mmol})} = 10.6_{(\text{g/kg})} \times \text{HIC}_{(\text{mg/g,dw})} \times \text{Body Weight}_{(kg)} + 55.85_{(\text{mg/mmol})}
\]
The change in TBIS between biopsies was calculated, and also divided by the number of days within the biopsy interval, providing an estimate of the mean daily change in TBIS (mgFe/kg/d).

**5.2.2 Interpretation of Iron Balance**

Ignoring dietary influences, no net change in the total body iron store (TBIS) indicates iron balance, a perfect balance between the amount of iron received through transfusion and the amount of iron eliminated through chelation therapy. In other words, chelation eliminated the same amount (moles) of iron from iron stores as was introduced through the processing of transfused blood, and the amount (moles) of iron in the TBIS over the interval did not change.

A net negative iron balance in TBIS indicates a reduction in the amount (moles) of stored body iron over an interval. This reflects the elimination of iron equivalent to the amount of transfusional iron received throughout the interval, plus the additional elimination of iron from body iron stores equivalent to the absolute change in the TBIS.

A net positive iron balance in TBIS indicates iron loading, as more iron is being incorporated into the iron store (through the processing of transfused RBCs), than is being eliminated through chelation therapy. The difference between the amount of transfusional iron received and the absolute increase of the TBIS, reflects the amount of storage iron that was removed by chelation therapy.

**5.2.3 Chelation Efficiency Calculation**

Our algorithm for calculating chelation efficiency is based on the assumption of a 1:1 chelator complex for iron:DFO (a hexadentate iron chelator; one molecule of DFO is capable of chelating 1 molecule of iron once it occupies all 6 valence/binding electrons of a ferric iron molecule) and a 1:3 chelator complex for iron:L1 (bidentate iron chelator; three L1 molecules are required to occupy all 6 valence/binding electrons of a ferric iron molecule). Therefore, chelation efficiency can be defined as how successful each mole
of DFO, or every 3 moles of L1, was at removing 1 mole of iron. Chelation efficiency is calculated by dividing the amount of iron that was removed from the body by the amount of chelation administered (the molar ratio of iron excretion to chelation administration, expressed as a per cent). By definition, the minimum chelation efficiency can only be 0%.

In patients with net negative iron balance:

Chelation Efficiency = \frac{(\text{amount of transfusional iron})_{\text{moles}} + |\text{change in TBIS}|_{\text{moles}} \times 100\%}{(\text{amount of chelation})_{\text{moles}} / (\text{molar ratio of chelator:iron complex})}

In patients with net positive iron balance:

Chelation Efficiency = \frac{(\text{amount of transfusional iron})_{\text{moles}} - |\text{change in TBIS}|_{\text{moles}} \times 100\%}{(\text{amount of chelation})_{\text{moles}} / (\text{molar ratio of chelator:iron complex})}

5.3 Statistical Analysis

Data are presented as means ± SE. Deferoxamine (DFO) and deferiprone (L1) data were compared using the two-sample t test (without the assumption of equality of variances) to reveal significant differences between the two groups when comparing means and distributions (Dixon, 1985; Pagano, 2000). The Fisher exact test was used to compare parameters reporting frequencies or ratios. The relation between chelation efficiency and initial hepatic iron concentration, initial molar amount of iron in the total body iron store, the rate of transfusional iron loading, and the dose of chelation were each determined with Pearson's coefficient of correlation and a linear regression analysis. All tests were two-tailed, using a P-value statistical significance level of 0.05.
5.4 Results

Sixteen adequate HIC intervals were obtained from twelve patients who were transfused and chelated with deferoxamine. The mean duration of these 16 intervals was 14.2 ± 0.7 (range: 10.4 – 20.0) months. For comparison, 31 adequate intervals were identified in 23 patients chelated with deferiprone (Table 5.2). The mean duration of these 31 intervals was 12.6 ± 0.6 (range: 8.2 – 19.5) months, which was statistically similar to the intervals in the DFO group (p=0.10).

5.4.1 Measures of the Amount of Transfusional Iron

Among DFO-chelated patients, the total amount of blood received through blood transfusions patients was 8716.9 ± 613.1 (range: 6158.0 – 15225.0) mL. The total volume of red blood cells (based on a hematocrit of 0.7), was 6101.8 ± 429.2 (range: 4310.6 – 10657.5) mL. The volume (mL) of red blood cells transfused is equivalent to the amount (mg) of transfusional iron administered, assuming that 1 mL of red blood cells contains 1 mg of iron. Considering the molecular weight of iron, the total amount of transfusional iron administered to DFO-chelated patients was 109.3 ± 7.7 (range: 77.2 – 190.8) mmoles. L1-chelated patients received 9347.8 ± 611.8 (range: 3517.0 – 19744.0) mL of transfused blood, equivalent to 6543.5 ± 428.3 (range: 2461.9 – 13820.8) mL of red blood cells. The molar amount of transfusional iron was 117.2 ± 7.7 (range: 44.1 – 247.5) mmoles. There was no statistically significant difference in transfusional iron between DFO- and L1-chelated patients (p=0.47)

5.4.2 Measures of the Amount of Chelation

DFO-chelated patients were 87.1 ± 2.8 (range, 60.0 – 98.6) % compliant with the prescribed dose of DFO, resulting in an actual mean daily DFO dose of 32.0 ± 2.3 (range, 10.6 – 46.5) mg/kg/day. Overall, the mean total molar amount of DFO administered during an interval was 854.0 ± 93.7 (range: 221.4 - 1505.6) mmoles.
<table>
<thead>
<tr>
<th></th>
<th>DFO</th>
<th>L1</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td># Patients (M/F)</td>
<td>12 (6 / 6)</td>
<td>23 (11 / 12)</td>
<td>0.99</td>
</tr>
<tr>
<td># Intervals (M/F)</td>
<td>16 (7 / 9)</td>
<td>31 (16 / 15)</td>
<td>0.76</td>
</tr>
<tr>
<td>Duration of Interval</td>
<td>14.2 ± 0.7 (10.4 – 20.0)</td>
<td>12.6 ± 0.6 (8.2 – 19.5)</td>
<td>0.10</td>
</tr>
<tr>
<td>Total Volume of Transfused RBC during Interval (mL)</td>
<td>6101.8 ± 429.2 (4310.6 – 10657.5)</td>
<td>6543.5 ± 428.3 (2461.9 – 13820.8)</td>
<td>0.47</td>
</tr>
<tr>
<td>Total Transfusional Iron during Interval (mmoles)</td>
<td>109.3 ± 7.7 (77.2 – 190.8)</td>
<td>117.2 ± 7.7 (44.1 – 247.5)</td>
<td>0.47</td>
</tr>
<tr>
<td>Total Amount of Chelation During Interval (mg)</td>
<td>561085.2 ± 61588.2 (145444.1 – 989191.8)</td>
<td>1397022.4 ± 96117.2 (400250 – 2703250)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total Amount of Chelation During Interval (mmoles)</td>
<td>854.0 ± 93.7 (221.4 – 1505.6)</td>
<td>10039.5 ± 690.7 (2876.4 – 19426.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HIC at Start of Interval (mg/g, dry weight)</td>
<td>7.7 ± 0.5 (2.8 – 11.6)</td>
<td>9.5 ± 0.7 (3.0 – 22.4)</td>
<td>0.06</td>
</tr>
<tr>
<td>HIC at End of Interval (mg/g, dry weight)</td>
<td>7.4 ± 0.8 (3.4 – 15.0)</td>
<td>11.7 ± 0.8 (5.3 – 20.1)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Change in HIC (mg/g, dry weight)</td>
<td>-0.3 ± 0.5 (-3.9 – 3.5)</td>
<td>2.2 ± 0.6 (-2.6 – 10.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>Change in [TBIS] (mg/kg)</td>
<td>-3.4 ± 5.7 (-41.3 – 37.1)</td>
<td>23.3 ± 6.6 (-27.6 – 108.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>Change in amount of iron in the TBIS (mmoles)</td>
<td>1.9 ± 5.7 (-34.4 – 63.2)</td>
<td>24.4 ± 5.6 (-33.7 – 86.1)</td>
<td>0.02</td>
</tr>
<tr>
<td># Intervals with reduced HIC or [TBIS]</td>
<td>11 / 16 (68.8%)</td>
<td>9 / 31 (29.0%)</td>
<td>0.01</td>
</tr>
<tr>
<td># Intervals with net negative iron balance</td>
<td>9 / 16 (56.3%)</td>
<td>9 / 31 (29.0%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Interval Change in Storage and Transf1l Iron (mmoles)</td>
<td>107.4 ± 9.7 (32.0 – 188.2)</td>
<td>92.8 ± 9.9 (-7.4 – 233.6)</td>
<td>0.54</td>
</tr>
<tr>
<td>Chelation Efficiency (%)</td>
<td>15.1 ± 2.4 (3.3 – 47.9)</td>
<td>2.6 ± 0.2 (0.0 – 4.8)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
L1-chelated patients took 93.6 ± 1.1 (range, 78.8 – 100.0) % of the prescribed dose, significantly more compliant than the DFO group (p=0.04). As a result, the mean daily L1 actually taken was 69.5 ± 1.1 (range, 59.5 – 86.8) mg/kg/day, and the mean total molar amount of L1 administered over an interval was 10039.5 ± 690.7 (range: 2876.4 – 19426.6) mmoles, statistically much greater than the amount of DFO (p<0.0001).

5.4.3 Measures of the Hepatic Iron Concentration

The mean initial hepatic iron concentration in both groups were statistically similar, and were both elevated beyond the 7 mg/g, dry weight threshold of increased risk of iron-related complications. Furthermore, a similar proportion of patients in both groups had initial hepatic iron concentrations exceeding 7 mg/gdw; 11 / 16 (68.8%) intervals in the DFO group compared to 23 / 31 (74.2%) in the L1 group (p=0.74).

A decrease in hepatic iron concentration was observed over 11 / 16 (68.8%) DFO intervals. Treatment with L1 was significantly less effective, as a reduction in hepatic iron concentration was observed in only 9 / 31 (29.0%) intervals (p=0.004). The final hepatic iron concentration in the DFO group was 7.4 ± 0.8 (range, 3.4 – 15.0).mg/g, dry weight, whereas in the L1- group the mean final HIC was significantly higher at 11.7 ± 0.8 (range, 5.3 – 20.1).mg/g, dry weight. The number of DFO-chelated patients with HICs above 7 mg/gdw at the end of therapy dropped to 7 / 16 (43.8%), compared to an increase to 25 / 31 (80.6%) of patients treated with L1 (p=0.02).

Therefore, DFO and L1 patients had statistically similar initial HICs. However, DFO patients managed to reduce their hepatic iron concentrations whereas in the L1 group, the HICs increased. After statistically similar periods of time, DFO patients ended with significantly lower hepatic iron concentrations compared to L1 patients.

5.4.4 The Total Body Iron Store and Iron Balance

Based on the formula derived by Angelucci et al (2000), all hepatic iron concentrations were converted to concentrations of the total iron stored in the body. Multiplying initial TBIS by the initial body weight calculates the initial number of moles
of iron in the total body iron store. Similarly, multiplying the final TBIS by the final body weight reveals the number of moles in the total body iron store at the end of the interval. The difference between the initial number of moles and the final number of moles defines net iron balance.

DFO patients initially had 55.0 ± 4.5 (range: 30.7 – 90.2) mmoles of total iron stored in their bodies, which increased to a final 56.9 ± 7.4 (range: 27.9 – 142.6) mmoles of iron. Therefore, DFO patients experienced a net positive iron balance, with a mean increase of 1.9 ± 5.7 (range: -34.4 – 63.2) mmoles of iron in the TBIS. However, this did not correspond with a similar mean increase in the concentration of the total body iron store (and hepatic iron concentration) as these DFO patients experienced enough of a mean gain in weight to more than accommodate the additional iron.

In comparison, L1 patients began with 89.7 ± 7.3 (range: 25.4 – 198.0) mmoles of total iron stored in their bodies, which increased to a final 114.1 ± 7.9 (range: 34.0 – 229.8) mmoles. This represents a mean increase of 24.4 ± 5.6 (range: -33.7 – 86.1) mmoles of iron into the total body iron store, which is a significantly greater net positive iron balance (p=0.02) than that observed in the DFO group. Furthermore, L1 patients did not gain enough weight to accommodate the additional iron. Therefore the concentration of their total body iron stores, and hepatic iron concentrations, unfortunately increased.

5.4.5 Chelation Efficiency

Neither iron chelator appears to be very efficient in removing iron from the body. The mean chelation efficiency calculated for DFO was 15.1% ± 2.4 (range: 3.3 - 47.9) %, while L1 was only 2.6% ± 0.2 (range: 0.0 - 4.8) % efficient at iron chelation. Nevertheless, DFO was significantly more efficient, and based on the observed mean chelation efficiencies, DFO appears to be greater than 5 times more efficient than L1 in iron chelation. Two patients chelated with L1 experienced 0% chelation efficiency, indicating that L1 was unable to chelate any iron from these patients.
Relation between Chelation Efficiency and Various Parameters

The DFO chelation efficiency among HCV+ patients was 11.7% ± 1.2 (range: 7.6 – 15.6) %, and among HCV- patients was 17.1% ± 3.7 (range: 3.3 – 47.9) %, revealing no significant difference (p=0.19). The chelation efficiency among L1-chelated HCV+ patients was 2.9% ± 0.2 (range: 2.2 – 3.5) %, while in the HCV- patients it was 2.6% ± 0.2 (range: 0 – 4.8) %, also revealing no significant difference (p=0.48). Within the DFO-chelated group, no significant correlation was observed between chelation efficiency and (i) the initial hepatic iron concentration (r=0.26, p=0.33), (ii) the initial size of the total body iron store (r=0.38, p=0.14), and (iii) the rate of transfusional iron loading (r=0.21, p=0.43). However, a very significant and fairly strong correlation was observed between chelation efficiency and the daily dose of DFO actually administered (r=0.79, p<0.0005) (Figure 5.1). Similarly in the L1-chelated group, the respective

![Figure 5.1. Correlation between the Dose of DFO Administered and the Chelation Efficiency.](image)
statistically insignificant correlations were (i) \(r=0.18, p=0.32\), (ii) \(r=0.14, p=0.44\), and (iii) \(r=0.26, p=0.16\). Just reaching statistical significance was the correlation between chelation efficiency and L1 dose administered, however the correlation was very poor \((r=0.36, p=0.04997)\).

5.5 Discussion

Measures of iron excretion have been used in the past to quantify the amount of iron removed from the body through chelation therapy. However, this technique of determining iron balance is greatly limited by the patients' lack of compliance with regular 24-hour urine and stool collection. Iron balance could also be calculated using indirect measures of the total body iron store based on hepatic iron and splenic iron concentrations measured with SQUID magnetic susceptibility, assuming that 90% of storage iron is contained within the liver and spleen of iron overloaded patients (Nielsen et al., 1995). However, the validity of this technique has not been established. Since Angelucci et al. (2000) showed a near perfect correlation \((r=0.98)\) between the total body iron store and the hepatic iron concentration, it is now possible to indirectly but accurately quantify the total body iron store using a direct reliable measure of the hepatic iron concentration. Therefore, the hepatic iron concentrations from serial liver biopsies can be used to calculate iron balance.

In regularly transfused patients receiving chelation therapy, net iron balance is achieved when the amount (moles) of transfusional iron put into the body is equivalent to the amount (moles) of iron removed through chelation therapy. However, it is important to realize how as a patient grows and their weight increases over an interval where net iron balance is achieved, the concentration of iron in the TBIS will decrease as the additional weight can also accommodate iron. In this case we would observe a reduction in the HIC. Similarly, if a patient's weight decreases over an interval where net iron balance is achieved, the concentration of their TBIS (and HIC) would increase. Therefore, a decrease in HIC and TBIS over time, does not necessarily correspond to a net negative iron balance. This helps to understand how net positive iron balance (when a patients has received more iron from transfusion than has been eliminated by chelation)
can still be observed in patients whose HIC has dropped while their body weight has increased. This explains the initially puzzling observation in our DFO group, of a mean slightly net positive iron balance, while these patients experienced a mean decrease in hepatic iron concentration. However, growth could not accommodate the relatively much larger amount of iron that accumulated into the iron stores of L1-chelated patients.

It has been well established that deferiprone is less effective at chelating iron than deferoxamine. Several studies have reported that at a dose of 75mg/kg/day, L1 is unable to eliminate enough iron from the body to achieve iron balance, and iron continues to accumulate beyond toxic concentrations (Hoffbrand et al., 1998; Olivieri et al., 1998; Tondury et al., 1998; Kontoghiorghes et al., 2000: Lancet correspondence). Hopefully, the toxicity of deferiprone should be clarified in the near future. Deaths have been reported in patients treated with L1, however the chelator could not be isolated as the cause (Agarwal et al., 1992; Mehta et al., 1991; Hoffbrand et al., 1998). These deaths were mainly from infections and the effects if iron overload including congestive heart failure. However, other causes of death were adult respiratory distress syndrome and systemic lupus erythematosus. Altogether, several of these deaths raised the suspicion of acquired immune deficiency in L1-treated patients. The incidence of neutropenia and agranulocytosis in L1-treated patients was identified at 5.4% and 0.6%, respectively (Cohen et al., 1998, 2000). Furthermore, 23% of patients in this series also experienced gastrointestinal symptoms and arthopathy, and 48% experienced a rise in ALT levels. A recent study, which included several of the patients in this study, has also identified that chelation with 75mg/kg/day of L1 over an extended period of time appears to worsen liver fibrosis (Olivieri et al., 1998). From this study, we observed net negative iron balance in just under 30% of patients who received up to 86.8 mg/kg/day of deferiprone. Based on the mean 2.6% L1 chelation efficiency observed in this study, we are able to estimate that 107.8 mg/kg/day of L1 would be required to achieve net iron balance in a patient transfused 10.5mL of red blood cells per kilogram body weight every 28 days. Until studies conclusively identify a lack of toxicity at L1 doses in this range, L1 should not be considered a safe and effective iron chelator in patients with thalassemia.

The safety and efficacy of deferoxamine have been thoroughly studied, and DFO remains the only iron chelator widely available for clinical use for the treatment of iron
overload. Several studies have identified that at clinically useful doses, deferoxamine can achieve iron balance. With the appropriate monitoring of body iron levels to guide prescription and avoid the toxicity of excessive iron chelation, DFO therapy has proven to be able to reduce iron overloaded body iron concentrations and maintain iron levels within safe ranges (between 3.2 - 7 mg/g, dry weight) (Olivieri, 1999). Olivieri et al. (1994) and Brittenham et al (1994) confirmed that the long-term complication-free survival of patients with thalassemia major could be achieved using DFO therapy. DFO prescribed at an average dose of 42mg/kg/day in patients receiving regular transfusions to maintain baseline hemoglobin between 8-9g/dL, could achieve iron balance, and reduce or maintain body iron concentrations below toxic levels (Brittenham et al., 1994). Notably, compliance with therapy ranged between 20 – 90%, suggesting that the actual mean dose administered to these patients was closer to a range between 8 – 38mg/kg/day. These patients appeared to be protected from cardiac disease, diabetes mellitus, and early death. Furthermore, a prescribed mean daily dose of approximately 50mg/kg/day appears to be effective at protecting against iron-induced endocrinopathies and increase the incidence of normal growth and sexual maturation, with 90% of patients regularly treated with chelation therapy since mid-childhood achieving normal puberty (Bronspiegel-Weintrob, 1990).

Another important feature of DFO therapy was identified by Barry et al.(1974), who revealed that low doses of DFO therapy (3g/week;weights not given) in thalassemia patients maintained on a high transfusion regimen, although insufficient to achieve iron balance in several patients, consistently arrested the progression of hepatic fibrosis.

Toxic effects of excessive deferoxamine therapy include ocular and auditory abnormalities (Olivieri et al., 1986; Porter and Huehns, 1989; Borgna-Pignatti et al., 1984; Orton et al., 1985; Rahi et al., 1986; Dickerhoff, 1987), sensorimotor neuropathy (Giardina et al., 1993), renal dysfunction (Koren et al., 1992), and pulmonary dysfunction (Freedman et al., 1990), which have been observed when doses exceed 50 mg/kg/day, and in patients with only mildly elevated hepatic iron concentrations (below 3.2 mg/gdw). It has recently been recommended that DFO dose not exceed 50mg/kg/day. Using the 15.1% chelation efficiency observed in DFO, we can estimate that 29.2 mg/kg/day of DFO is required to achieve net iron balance in a patient transfused 10.5mL
of red blood cells per kilogram body weight every 28 days, well below the recommended 50mg/kg/day threshold. Furthermore, calculations for the individualized prescription of DFO are provided (see Section 5.5.3 and 5.5.4).

Chelation efficiency

Chelation efficiency reflects an iron chelator's ability to reach intracellular and extracellular iron stores, compete with other iron ligands, and be excreted by the body. No previous study has quantitatively reported the efficiency of an iron chelator in removing iron from the body. While the amounts of transfusional iron and chelation have been identified, a calculation of chelation efficiency must include an accurate measure of the amount of iron that was removed through chelation. Several studies have focussed on iron balance, identifying the excretion of iron in the urine and stool, however chelation efficiency is not reported and the data published cannot be appropriately analyzed. The only report on iron balance that provides appropriate data to calculate chelation efficiency, involved 4 regularly transfused patients with sickle cell disease, who were chelated for a period with L1 (75 mg/kg/d) then DFO (50mg/kg/d) with near perfect compliance (Collins et al., 1994). In this cross-over dose response study, based on measures of total iron excretion, a mean chelation efficiency of 20.7% ± 0.7 (range: 19.3 – 22.4)% was observed using DFO, while the mean chelation efficiency observed using L1 was 5.2% ± 0.9 (range: 3.9 – 7.8)%

In comparison to the results of our study, slightly better chelation efficiency was identified with both chelators, perhaps reflecting different characteristics of iron storage in sickle cell disease. Furthermore, a 4-fold better chelation efficiency of DFO compared to L1 was evident in this group, compared to our observed 5-fold better chelation efficiency of DFO.

The initial body iron concentration, the rate of iron accumulation, and the dose of chelation therapy have all been suggested as potentially influencing the efficacy of chelation therapy (Kontogiorghes et al., 2000). Notably, HCV infection does not appear to affect the chelation efficiency of either drug. From our data, the only correlation identified in the L1 group was a barely significant but nevertheless very weak correlation between chelation efficiency and the dose of L1 administered. However, there may not
be enough variation in the doses of L1 administered to establish a representative correlation. Similarly, the lack of correlation with initial hepatic iron concentration may also be misleading, since we may not have a broad enough range in initial hepatic iron concentrations. A more appropriate correlation would include a wider range in HIC, from mildly to massively overloaded patients. In our series, only 3 initial hepatic iron concentrations were above 15 mg/g, dry weight. Therefore, it may be inappropriate to comment further on this lack of correlation.

As in the L1 group, no correlation between initial HIC and chelation efficiency was observed, but this could again be due to the small range of initial HICs, with 12/16 (75%) initial HICs between 6-9 mg/gdw, and a maximum HIC of only 11.6mg/gdw. In the DFO group, the only variable that significantly correlated with chelation efficiency, was the dose of DFO actually administered (not the prescribed dose). The inverse relationship suggests that DFO initially removes an easily accessible fraction of iron, and once this fraction is exhausted any additional DFO (at greater doses) attempts to chelate iron from less accessible sources. Therefore, DFO appears to be able to easily chelate iron from certain sources, while struggling with iron from other sources.

It appears reasonable to suggest that the most accessible iron fraction for DFO chelation would be non-transferrin bound iron (NTBI), and the intracellular labile iron pool (Hershko et al., 1998). As transferrin becomes completely saturated after a period of regular transfusions, intracellular NTBI and extracellular “non-transferrin-bound plasma iron” (NTBPI) species begin to appear. Both are likely the primary, relatively “easy” targets for chelation. Furthermore, the intracellular labile iron pool includes the iron in transit from the transferrin receptor to ferritin molecules, which temporarily exists in a weakly bound low molecular weight complex accessible to DFO (Hershko et al., 1998). However, once these fractions are removed, DFO may have a much more limited ability to remove iron from other extracellular and intracellular iron ligands and stores, and the chelation efficiency would decrease accordingly.

A remarkably high chelation efficiency (47.9%) was in fact observed in a patient treated with DFO. The patient had an initial HIC of 6.2mg/g, dry weight, which justified iron chelation, however hearing loss and growth retardation was considered to be secondary to DFO toxicity. Therefore, the patient’s DFO dose was reduced to
approximately 10.6mg/kg/d. The patient still achieved net negative iron balance and a drop in HIC within a year, to 3.6 mg/g, dry weight. Liver biopsies also revealed no hepatic fibrosis. This drop in HIC using so little DFO is not well understood, although the high chelation efficiency may be reasonable.

Since its development, it was known that L1 did not have the capability to be an efficient iron chelator. However, the potential clinical applications of this drug relied on its ability to be safely administered at enormous doses, to counteract the poor chelating efficiency. Due to its small size, L1 can be administered orally, is rapidly absorbed, and appears in the blood within minutes (Kontoghiorghes et al., 1990). Factors contributing to the poor efficiency of L1 include the fact that:

1. L1 is a bidentate ligand requiring three molecules to complex with iron
2. L1 is rapidly glucuronidated as it enters the blood, which affects it’s iron affinity
3. L1 is not very successful in competing for ferric iron with transferrin, a major iron source for chelation by L1
4. L1 has a short elimination half-life of 91.1 ± 33.1 minutes, resulting in a very limited length of time where the concentration of deferiprone in the blood is high enough for 3 molecules to complex with iron (Hider et al., 1999: BIOIRON'99 abstract).

As a result, L1 struggles to complex with extracellular iron, and is excreted (almost exclusively) via the kidneys (Olivieri and Brittenham, 1997).

According to the results from this study, deferoxamine is five times more efficient than deferiprone, but is also an inefficient iron chelator. The major limitation is deferoxamine’s rapid metabolism in plasma, with a half-life of less than 15 minutes (Summers et al., 1979). The chelator has a very short window of time to bind iron, and as a result most of the chelator passes unbound into the urine. Although DFO cannot independently remove iron from transferrin, the stability constant of ferrioxamine (iron bound to desferrioxamine) is $10^{31}$, actually exceeding that of diferric transferrin. DFO relies on other chelating compounds (ie citrate) to dislodge iron from transferrin, which also influences it’s chelation efficiency (Turcot et al., 2000). Deferoxamine is a hydrophilic iron chelator, limiting its accessibility to intracellular iron stores. However,
DFO can chelate iron from stores within plasma macrophages after the catabolism of red cells, and ferrioxamine is eventually excreted in the urine, and can also penetrate into hepatocytes and results in chelation of cytosolic iron and subsequent biliary excretion into the feces (Hershko et al., 1998).

In summary, chelation efficiency represents the proportion of the total amount of iron chelator administered that actually reaches body iron stores, binds to iron, and is eliminated from the body. L1 is a much less efficient iron chelator than DFO, and more importantly, does not seem capable of achieving net iron balance at safe doses in regularly transfused patients with β-thalassemia. This study was the first to quantify the chelation efficiency of DFO and L1, which at clinically used doses appears to be approximately 15.1% and 2.6%, respectively. Together with the amount of transfusional iron, the chelation efficiency can be used to calculate the dose of iron chelation required to achieve net iron balance, and target safe body iron concentrations.

5.5.1 Limitations

The increased metabolism of ascorbate causing ascorbate deficiency has been identified in thalassemia patients with iron overload (O’Brien, 1974; Chapman et al., 1982). Ascorbic acid, administered to all our DFO-chelated patients, may have the ability to solubilize iron into a chelatable form accessible to deferoxamine (Hussain, 1977; Bridges and Hoffman, 1986). The toxicity of ascorbate supplementation during L1 therapy is unknown, and L1 patients did not receive supplementation. Therefore, ascorbate supplementation would be a confounding influence on chelation efficiency. DFO chelation efficiency may be more appropriately referred to as DFO+VitC chelation efficiency. A more accurate L1 to DFO comparison would either include vitC supplementation to L1 chelation, or compare L1 chelation efficiency to the chelation efficiency of DFO in patients not receiving ascorbate supplementation.
5.5.2 Future Direction

In order to better understand the chelation efficiency of deferoxamine, investigators must more appropriately define its mechanism of iron chelation, determining how effective DFO is at removing iron from parenchymal vs RE cell stores. One suggestion is to analyze the liver iron distribution from the liver biopsies of these patients, and study the change in both iron concentration and iron distribution in these livers over the intervals of chelation. This could identify if DFO preferably chelates iron from RE cell stores vs parenchymal cell stores, and the amount of iron that is removed from each cell store.
5.5.3

Quantitative Individualized Deferoxamine Prescription To Achieve Iron Balance

In almost every case, the patient will present at clinic for a scheduled transfusion. Requirements:

- Current body weight
  - Ideally this should actually be an estimate of their projected mean weight until the next clinic visit.
- The volume of blood actually transfused this visit
  - Or the volume to be transfused based on the prescribed cc/kg.
- The number of days until the next scheduled transfusion.
- The patient's DFO compliance since the last visit.

Step 1. Calculate the amount of transfusional iron received this visit.

\[ \text{Tx-RBC}_{(mL)} = (\text{Total volume}_{(mL)} \text{ of blood transfused}) \times (\text{Hematocrit of transfused blood}) \]

\[ \text{Tx-Fe}_{(mg)} = \text{Tx-RBC}_{(mL)} \]

\[ \text{Tx-Fe}_{(mmoles)} = (\text{Tx-Fe}_{(mg)}) / 55.85_{(mg/mol)} \]  
  [Using MW of iron = 55.85g/mol]

Step 2. Calculate the amount of DFO needed to remove this iron.
(based on the newly identified DFO chelation efficiency of 15.1%).

\[ \text{DFO}_{(mmoles)} = (\text{Tx-Fe}_{(mmoles)}) / 0.151 \]

\[ \text{DFO}_{(mg)} = (\text{DFO}_{(mmoles)}) \times 656.8_{(mg/mmol)} \]  
  [Using MW of DFO = 656.8 g/mol]

Step 3. Calculate the daily dose required (mg/kg/day).

Dose required_{(mg/kg/day)} = (\text{DFO}_{(mg)} \text{ needed}) / (\text{Patient weight}_{(kg)}) / (\text{days until next visit})

Step 4. Calculate prescription based on last month's compliance.

\[ \text{Prescribed dose}_{(mg/kg/day)} = (\text{Dose required}_{(mg/kg/day)}) / (\text{Compliance}) \]  
  [ie. 50% compliance = 0.5]
Quantitative Individualized Deferoxamine Prescription To Reduce the Concentration of the Total Body Iron Store and the Hepatic Iron Conc.

In this situation, an elevated HIC would be identified, and the goal of chelation therapy would be to reduce the HIC to a more acceptable pre-selected level, over the next year. The strategy would be not only to achieve net iron balance, as above, but to also remove additional iron from the total body iron store.

At the next clinic visit for a scheduled transfusion, since receiving the patients HIC from the iron lab:

Requirements:
- Date of recent biopsy or SQUID
- HIC from SQUID or liver tissue weighing >1.0mg, dry weight
- Current body weight
- Transfusion strategy (cc/kg and interval)
- The number of days until the next scheduled transfusion/visit
- The patient’s DFO compliance since the last visit

Step 1. Determine the initial amount of iron in the patient’s total body iron store.

Initial TBIS\(_{(mg)}\) = HIC\(_{(mg/g,dw)}\) x 10.6\(_{(g/kg)}\) x Body weight\(_{(kg at biopsy/SQUID)}\)

Initial TBIS\(_{(mmoles)}\) = Initial TBIS\(_{(mg)}\) / 55.85\(_{(MW of Iron)}\)

Step 2. Determine the target amount of iron for the patient’s total body iron store in 1 year.

Target TBIS\(_{(mg)}\) = Target HIC\(_{(mg/g,dw)}\) x 10.6\(_{(g/kg)}\) x Estimated Body weight\(_{(kg in 1 year)}\)

Target TBIS\(_{(mmoles)}\) = Target TBIS\(_{(mg)}\) / 55.85\(_{(MW of Iron)}\)

Step 3. Calculate the number of moles of iron to be eliminated from the TBIS (in addition to net iron balance) in order to achieve the desired drop in HIC in 1 year.

Required reduction in TBIS\(_{(mmoles)}\) = Initial TBIS\(_{(mmoles)}\) - Target TBIS\(_{(mmoles)}\)

Required daily reduction in TBIS\(_{(mmoles/day)}\) = Reduction in TBIS\(_{(mmoles)}\) / 365 days
Step 4. At this and every clinic visit until the next HIC:
Calculate the amount of transfusional iron received this visit.

\[
Tx-RBC_{(mL)} = (\text{Total volume}_{(mL)} \times \text{Hematocrit of transfused blood})
\]

\[
Tx-\text{Fe}_{(mg)} = Tx-RBC_{(mL)}
\]

[1mL of red blood cells contains 1mg of iron]

\[
Tx-\text{Fe}_{(mmoles)} = (Tx-\text{Fe}_{(mg)}) / 55.85_{(mg/mmol)}
\]

[Using MW of iron = 55.85g/mol]

Step 5. Calculate the amount of DFO needed to remove:
\(\text{a. An equivalent amount of iron (as the transfusional load) to achieve iron balance.}\)
\(\text{b. The additional moles of iron that must be removed daily to achieve our goal. (based on the newly identified DFO chelation efficiency of 15.1%).}\)

\(\text{a. DFO}_{(mmoles)} \text{ to achieve iron balance} = \frac{Tx-\text{Fe}_{(mmoles)}}{0.151}\)

\(\text{b. Additional DFO}_{(mmoles)} \text{ to achieve target} = \frac{(\text{Required daily reduction in } \text{TBIS}_{(mmoles)} \times \# \text{ of days until next visit})}{0.151}\)

\[\text{Total DFO}_{(mg)} \text{ needed until next visit} = (\text{DFO}_{(mmoles)} \text{ to achieve iron balance} + \text{Additional DFO}_{(mmoles)} \text{ to achieve target}) \times 656.8\]

[MW of DFO = 656.8 g/mol]

Step 6. Calculate the daily dose required (mg/kg/day).

\[\text{DFO dose required} \text{ (mg/kg/day)} = \frac{\text{Total DFO}_{(mg)} \text{ needed until next visit}}{\text{Patient weight}} / \text{(days until next visit)}\]

Step 7. Calculate prescription based on last month’s compliance.

\[\text{Prescribed DFO dose} \text{ (mg/kg/day)} = \frac{\text{DFO dose required}_{(mg/kg/day)}}{\text{Compliance}} \text{ [ie. 50% compliance = 0.5]}\]
Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

Hepatic iron concentration, quantified biochemically using a sample of liver tissue weighing greater than 1.0mg (dry weight), or quantified using SQUID magnetic susceptometry, accurately reflects the total amount of iron stored within the body (Angelucci et al., 2000; Brittenham et al., 1982, 1988, 1993; Fischer et al., 1999). This thesis emphasizes the importance and usefulness of hepatic iron quantitation in the management of regularly transfused haemoglobinopathy patients. These measures assure that clinicians are well-informed when making decisions on the initiation of chelation therapy, and when obtained periodically, provide accurate monitoring of body iron levels for clinicians to base decisions on adjustments to chelation therapy.

6.1 Ultrasound-Guided Liver Biopsies in Haemoglobinopathy Patients

The results from the liver biopsy report reveal that percutaneous liver biopsy is a very safe procedure, with no mortality and a complication rate of less than 1% experienced after more than ten years and 911 biopsies. Ultrasound-guidance appears to have significantly improved the technique, and the procedure has been tremendously successful at acquiring tissue, with over 80% of biopsies over the last 3 years capturing enough tissue for both accurate iron quantitation and histological assessment. Almost all the remaining biopsies, although less successful, are not unsuccessful, as they do obtain enough tissue for one of the two analyses.

Another clear message delivered through the biopsy report is that a serial biopsy system can be successfully incorporated into a haemoglobinopathy program. Serial liver biopsies have been performed in over 200 patients for monitoring body iron levels, with patients biopsied as much as 8 times over the 10-year period. Patients appear to have accepted periodic biopsies as part of their clinical care, after being informed of the poor reliability of serum ferritin concentrations, and realizing that the procedure, although uncomfortable, is relatively non-invasive and is the ideal method of monitoring body iron
levels. Our results should encourage clinicians to incorporate a serial liver biopsy system into their hemoglobinopathy programs to monitor body iron levels, also realizing the opportunity to assess and monitor the liver histologically. Our suggestion would be to have liver biopsies performed percutaneously with ultrasound-guidance by an experienced operator or interventional radiologist, often using conscious sedation to improve patient comfort.

6.2 The Rate of Iron Accumulation and the Initiation of Chelation Therapy

After identifying the rate of iron accumulation in regularly transfused patients with sickle cell disease and thalassemia, it has become evident that dangerously elevated hepatic iron concentrations are achieved within the first 1-2 years of transfusion therapy in both disorders. Therefore, clinicians should use hepatic iron concentrations to identify body iron levels after a period of approximately 12 months of transfusions, to confirm if patients have achieved elevated iron levels justifying the initiation of chelation therapy. Furthermore, clinicians would be able to identify patients with relatively normal iron levels, in whom chelation therapy would be premature, exposing the patient to the risks of DFO toxicity. Currently most clinicians rely on measures of the serum ferritin concentration when considering chelation therapy. However, our results confirm several other reports that these measures are unreliable, and should not be used to guide chelation therapy, especially in patients with sickle cell disease.

Histological analysis of liver biopsy tissue also recognized similar patterns of iron deposition among regularly transfused patients with sickle cell disease and thalassemia. Combined with the similar rates of iron accumulation, we would anticipate that patients with sickle cell disease develop complications secondary to iron overload similar to those observed at elevated hepatic iron concentrations in thalassemia. Therefore, patients with sickle cell disease receiving regular transfusions should receive the same treatment as regularly transfused patients with thalassemia.
6.3 Monitoring Iron Levels and Achieving Iron Balance with Chelation Therapy

Periodic hepatic iron concentrations provide clinicians with the most accurate method of monitoring iron levels, identifying if patients’ body iron levels are increasing or decreasing during chelation therapy. Currently, adjustments to chelation therapy are based on fluctuations in the serum ferritin concentration, or more appropriately on fluctuations in hepatic iron concentration when available. However, as a result of the chelation efficiency study, clinicians now know the efficiency of DFO chelation (the number of moles of DFO required to remove 1 mole of iron), and can for the first time calculate the appropriate doses to achieve iron balance. Furthermore, hepatic iron concentrations now provide a reliable estimate of the total amount of storage iron in the body (Angelucci, 2000). Clinicians can combine this measure with the 15% chelation efficiency of DFO, to calculate the dose of chelation required to reach specific target hepatic iron concentrations.

The chelation efficiency study also confirmed that deferoxamine chelation is capable of achieving iron balance in regularly transfused patients with thalassemia, and patients experienced a mean decrease in hepatic iron concentration. Deferiprone is five times less efficient at iron chelation than deferoxamine, and does not appear to be capable of achieving iron balance, even using relatively high doses, well beyond those suspected to be toxic.

Overall, the results of this thesis should have various implications on the treatment of regularly-transfused patients with sickle cell disease and thalassemia, after clarifying the rate of iron accumulation and offering quantitative techniques for identifying and better controlling the progression of iron overload using chelation therapy.
REFERENCES


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