

New targeted therapies and diagnostic methods for iron overload diseases

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1. ABSTRACT

Millions of people worldwide suffer from iron overload toxicity diseases such as transfusional iron overload in thalassaemia and hereditary haemochromatosis. The accumulation and presence of toxic focal iron deposits causing tissue damage can also be identified in Friedreich's ataxia, Alzheimer's, Parkinson's, renal and other diseases. Different diagnostic criteria of toxicity and therapeutic interventions apply to each disease of excess or misplaced iron. Magnetic resonance imaging relaxation times T2 and T2* for monitoring iron deposits in organs and iron biomarkers such as serum ferritin and transferrin iron saturation have contributed in the elucidation of iron toxicity mechanisms and pathways, and also the evaluation of the efficacy and mode of action of chelating drugs in the treatment of diseases related to iron overload, toxicity and metabolism. Similarly, histopathological and electron microscopy diagnostic methods have revealed mechanisms of iron overload toxicity at cellular and sub-cellular levels. These new diagnostic criteria and chelator dose adjustments could apply in different or special patient categories e.g. thalassaemia patients with normal iron stores, where iron deficiency and over-chelation toxicity should be avoided.

2. INTRODUCTION

Iron is an essential metal found in humans and all other living organisms, playing an important role in many physiological processes including normal growth and development. Under normal conditions iron absorption, transport, storage, utilisation, recycling

and excretion are genetically controlled by regulatory metabolic pathways, homeostatic mechanisms and related regulatory proteins (1–3). Despite the strict regulatory control of iron, the abnormalities of iron metabolism are very common, affecting millions of people worldwide (1–3).

It is estimated that about a third of the human population is affected at some stage in their life by iron metabolic disorders and particularly by iron deficiency. Many millions of people also suffer from iron overload diseases such as hereditary haemochromatosis and thalassaemia, both of which are caused by a genetic malfunction. Iron overload in hereditary haemochromatosis is caused by increased gastrointestinal iron absorption and in thalassaemia by haemoglobin iron from chronic red blood cell transfusions (1–3).

In addition to the essential role of iron in many biological processes, iron also plays an important catalytic role in free radical pathology and oxidative damage, which is observed in tissue damage in almost all major iron loaded and also non iron loaded diseases including cardiovascular, neurodegenerative, hepatic and renal diseases, as well as in cancer and ageing (4–5).

Many of the disease models related to iron metabolic abnormalities appear to be affected mainly by genetic, regulatory and iatrogenic factors. Within this context and in addition to iron deficiency and iron overload, there are many other acquired and hereditary conditions with abnormal iron distribution

leading to body iron imbalance and in many cases specific tissue iron accumulation. For example, in the anaemia of chronic disease which is observed in many chronic inflammatory conditions, iron is mostly stored in the cytoplasm of reticuloendothelial macrophages (1,2,6,7). There are also many other diseases of abnormal iron deposition in addition to the anaemia of chronic disease which originate from similar genetic, regulatory and iatrogenic factors but with different clinical implications. For example, an increase in iron accumulation and deposition is observed in the mitochondria of patients with sideroblastic anaemia and Friedreich's ataxia (7–9).

Under normal conditions, the physiological parameters of iron metabolism are generally characterised by the normal functioning of pathways and metabolic activity of iron-containing proteins, including physiological concentration levels of iron biomarkers such as haemoglobin, serum ferritin, serum iron, plasma transferrin saturation, as well as the level of stored iron in the liver and other organs. The levels of these iron biomarkers are the main regular parameters measured in clinical laboratories for the diagnosis of iron metabolic abnormalities. Similarly, the recent introduction of magnetic resonance imaging (MRI) relaxation times T2 and T2* are routinely used by MRI diagnostic centres for monitoring iron deposits in iron loading diseases, as well as in conditions with focal or localised iron deposits (8–12).

Regarding therapeutic approaches, it is currently feasible to treat most of the diseases related to iron metabolic imbalance using established and effective methods. For example, iron supplementation is widely used and is effective in most cases for the treatment of iron deficiency anaemia. Similarly, venesection is also widely used and is effective in most cases for the treatment of hereditary haemochromatosis. However, iron overload in thalassaemia is more difficult to treat and can only be accomplished using chelation therapy and the same seems to apply for the removal of focal or localised iron deposits in Friedreich's ataxia, Parkinson's disease and also other categories of patients (13). Major difficulties are also observed in the treatment of the anaemia of chronic disease in many conditions such as cancer, rheumatoid arthritis and haemodialysis, where oral or intravenous iron, with or without erythropoietin combination may be used.

The different forms of treatment related to iron overload, as well as other iron metabolic disorders are routinely monitored using the iron biomarkers and a number of diagnostic techniques such as MRI, histopathology of liver and bone marrow biopsies etc. Similarly, the extent of organ damage and the progress of the treatment of iron overload and other iron metabolic disorders can also be monitored by

a number of other diagnostic methods which are related to organ function such as liver enzyme levels, creatinine clearance levels and also techniques such as echocardiography and ultrasound (13).

3. DISEASES OF IRON OVERLOAD

Under normal conditions body iron balance in humans is maintained because of equivalence in the amount of iron intake from dietary iron absorption in comparison to iron excretion. Despite there being an increased capacity for the storage of excess iron in the human body, most of the iron is reutilised and there is no major regulatory pathway or mechanism for the rapid excretion of iron. Within this context there are many, varied diseases of gross body iron overload where excess iron intake, accumulation, storage and deposition can result in excess body iron burden, causing iron related toxicity and damage to various tissues and organs (1–3). Similarly, there are many other diseases where body iron levels and iron related biomarkers are generally within the normal physiological ranges but focal or localised deposited iron in the form of polynuclear iron complexes, including ferritin aggregates and haemosiderin, as well as other forms of iron can cause toxicity, molecular, cellular and tissue damage and abnormalities in the normal function of organs (4,5,7–9).

The causes of the gross body or focal excess iron accumulation and the resulting toxicity are usually related to an inherited condition or abnormality in a specific iron metabolic pathway or protein. In particular, increased iron absorption and repeated red blood cell transfusions or the combination of these two processes are the main pathways causing diseases of excess body iron burden. Accordingly, the most common iron loading diseases are hereditary haemochromatosis, where excess body iron load is derived from increased gastrointestinal absorption of dietary iron and thalassaemia, where excess body iron load is caused mainly from regular red blood cell transfusions and also to a lesser extent by increased iron absorption (1,2,13).

The disease with the highest mortality and morbidity rate related to iron overload and of any other iron metabolic disorders worldwide is thalassaemia, which is found mainly in developing countries of South East Asia, the Middle East and the Mediterranean. In these geographical regions the heterozygote carrier rate is high, for example 1 in 6 individuals in Cyprus is heterozygote carrier of thalassaemia. It is also estimated that more than 100000 thalassaemia babies are born worldwide every year with 9000 in India alone (13–15).

There are many types of thalassaemia affecting the production of the alpha, beta, gamma,

delta and epsilon globin chains of haemoglobin (13,14). The most common thalassaemia disorder is beta-thalassaemia, where there is a reduced or absent production of the beta globin chain of haemoglobin in the red blood cells. Despite that the alpha globin chain in beta-thalassaemia patients is produced normally, the absence of beta chains cause alpha chains to precipitate and damage to the red blood cells. As a result of this abnormality, the red blood cells in beta-thalassaemia patients are non functional resulting in severe anaemia and ineffective transport of oxygen to the tissues. Patients with beta-thalassaemia, as well as many other categories of patients with refractory anaemias can only survive if they are regularly transfused throughout their whole lives with normal red blood cells in order to maintain haemoglobin levels usually greater than 9 mg/dL (13).

There are many problems associated with the treatment of thalassaemia patients. In particular, most thalassaemia patients are born in developing countries and die without treatment because of the absence of health facilities and the high expenditure of regular transfusions and drug treatments (5). Life expectancy is low in the absence of treatment and usually non-transfused thalassaemia patients die due to ineffective erythropoiesis by the age of 7 years. Life expectancy increases in regularly transfused thalassaemia patients. The rate of transfusion in these patients is variable and depends on age, weight, spleen size, red cell antibodies and other parameters (13). Usually it ranges from about 1–3 units of packed red blood cells (1 unit, is about 200 ml = 200 mg of iron) every 1–4 weeks. This rate of transfusion causes on average a net iron intake and deposition in the body of about 15–20 mg/day. An additional amount of dietary iron of up to 6 mg/day can also be deposited in the body from increased gastrointestinal iron absorption, especially when the patients have increased haemopoietic activity because of the underlying anaemia. However, if chelation therapy is not available the regularly transfused thalassaemia patients die usually by the age of 20 years, mainly from congestive cardiac failure due to cardiac iron overload toxicity (16,17). Iron overload toxicity in thalassaemia is manifested in many other organs in addition to the heart, including the liver, spleen, pancreas and other endocrine organs (13–17). In contrast, the life expectancy of thalassaemia patients receiving chelation therapy increases substantially and many patients adhering to the chelation protocol with deferiprone (L1) and deferoxamine (DF) and their combination can now live beyond the age of 50 years (Figure 1) (13).

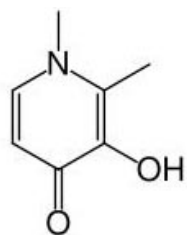
Iron chelation therapy is widely used in many transfusional iron overloading conditions in addition to thalassaemia, such as sickle cell anaemia, myelodysplasia, myelofibrosis, aplastic anaemia, sideroblastic anaemia, Blackfan Diamond anaemia,

Fanconi's anaemia, hereditary hypochromic anaemia, haemodialysis, different types of cancer etc. The toxic side effects of iron overload and the chelation therapy strategies in these conditions are similar to those observed in iron loaded thalassaemia patients. Within this context, the aim of the chelation therapy in all these conditions including thalassaemia is to remove sufficient amounts of iron (about 15–25 mg/day) in order to maintain negative iron balance compared to iron accumulated in the body mainly from transfusions. Overall, the ultimate and main aim of chelation therapy in thalassaemia and the other transfusional iron loaded conditions is to achieve and maintain normal body iron levels.

Hereditary haemochromatosis is the main condition of iron overload caused by increased gastrointestinal dietary iron absorption (18,19). Under normal conditions the maintenance of body iron balance in normal individuals is achieved because the rate of iron absorption is equivalent to the rate of iron excretion and other bodily iron losses. In contrast, in hereditary haemochromatosis the rate of iron loading from increased gastrointestinal dietary iron absorption can be much faster than the rate of iron excretion. Consequently, in the absence of a mechanism of rapid excretion of excess iron in humans, iron overload develops gradually and at certain levels it causes damage to the liver and other organs.

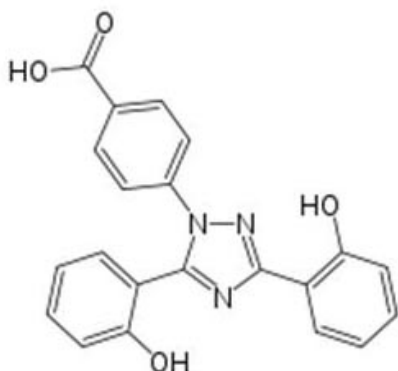
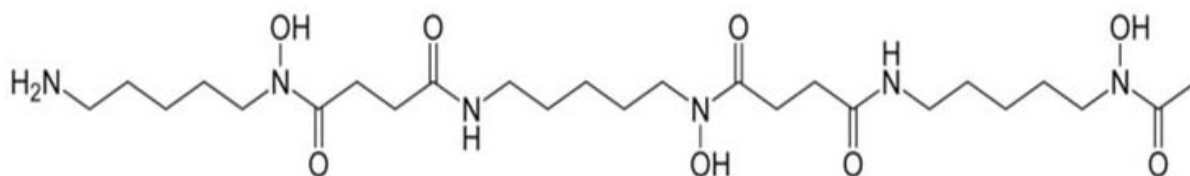
Iron overload in hereditary haemochromatosis patients is generally related to the presence of the HFE gene and the C282Y mutation (18, 19). Hereditary haemochromatosis is the most common genetic disorder among the Caucasian population in the USA and Northern Europe. The frequency of the homozygous type for the haemochromatosis mutation in these areas is estimated to occur in 1 in about 300 persons and at least 1 in 10 persons is a heterozygote carrier (18–20). It should be noted that not all hereditary haemochromatosis patients have the C282Y mutation and the severity of the iron loading and toxicity levels is variable among those affected by having or not having the mutation (18–20).

Several other types of mostly genetic iron overloading conditions caused by increased gastrointestinal iron absorption are known in addition to hereditary haemochromatosis. These include thalassaemia intermedia and many other non-transfusion dependent thalassaemias, attransferrinaemia and aceruloplasminaemia. In all these other conditions the excess iron is absorbed from the diet in the gastrointestinal tract, which usually on average contains about 6 mg iron per day. It is estimated that in contrast to the rate of iron absorption in normal individuals which is about 1–1.4 mg per day, in hereditary haemochromatosis patients the rate of iron absorption is several fold higher than in normal

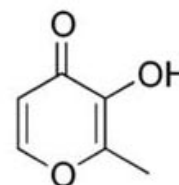


Deferiprone

Deferoxamine



Deferasirox



Maltol

Figure 1. The chemical structure of the iron chelating drugs. Deferiprone (L1), deferoxamine (DF) and deferasirox (DFRA) are worldwide used for the treatment of thalassaemia and other transfusional iron loading conditions. The maltol iron complex is used for increasing iron absorption in iron deficiency anaemia.

individuals and body iron overload develops from excess dietary iron absorption and accumulation over many years. In most cases of hereditary haemochromatosis patients, the absence of early diagnosis and therapy causes the progressive development of iron overload which is usually detected after the age of 40–50 years. In these cases the detection of iron overload is usually due to organ damage as a result of the toxic side effects of the accumulation of excess iron deposition mainly in the liver but also in other organs. In this context, organ damage can be prevented if the condition is diagnosed and appropriately treated much earlier in life (18–21).

Iron overload in hereditary haemochromatosis patients is treated by phlebotomy or venesection through the removal of iron in the form of haemoglobin present in red blood cells. Regular blood removal in

hereditary haemochromatosis patients usually begins after the age of 18 years (18–21). During phlebotomy treatments there is a physiological need by the body to overcome the induced anaemia, by increasing the haemopoietic activity of the bone marrow and for replenishing lost red blood cells. In this context, excess iron is slowly mobilised from the liver and diverted to the erythropoietic tissues of the bone marrow to be utilised in the production of haemoglobin in young red blood cells. The level of blood removal by phlebotomy depends on the iron load of patients. For example in heavily iron loaded patients 400–500 ml of blood, which is equivalent to 200–250 mg iron can be removed every week. In non-heavily iron loaded hereditary haemochromatosis patients, the same amount of blood can also be removed every 1 to 3 months and used for iron balance maintenance therapy. During

phlebotomy treatments the haemoglobin levels are usually maintained above 11 g/dL for safety reasons. This therapeutic procedure prevents iron accumulation from increased gastrointestinal iron absorption and also the iron overload toxicity in the liver and other organs. Similarly, it can also effectively promote the maintenance of normal iron status in hereditary haemochromatosis patients (18–21).

Overall it appears that genetic, dietary, iatrogenic, immunological and other factors can influence the rate of iron overload and related toxicity in both transfusional iron loading conditions such as thalassaemia, sickle cell anaemia, myelodysplasia and also iron overload from increased gastrointestinal iron absorption such as in hereditary haemochromatosis, thalassaemia intermedia and many other non-transfusion dependent thalassaemias.

4. DISEASES RELATED TO IRON TOXICITY IN PATIENTS WITH NORMAL IRON STORES

There are many diseases in addition to transfusional iron overload and hereditary haemochromatosis, where abnormalities of iron metabolism and excess iron deposition can cause different clinical complications. Most of these abnormalities originate from inherited, environmental, iatrogenic and metabolic imbalance factors. In some of these diseases iron may be deposited in specific organelles, cells, organs or tissues, despite that the general iron related indices such as serum ferritin and transferrin iron saturation are within the normal physiological range.

In particular, an increase in iron accumulation and deposition in the form of polynuclear iron is observed in the mitochondria of Friedreich's ataxia and sideroblastic anaemia patients (13,22,23). In contrast, no excess iron deposition is observed in the mitochondria of iron loaded thalassaemia or hereditary haemochromatosis patients (13). Despite that iron can be diverted and cause mitochondrial iron deposition and anaemia in sideroblastic anaemia patients, no anaemia or abnormal serum iron or serum ferritin levels are generally observed in Friedreich's ataxia patients (13,22,23).

Friedreich's ataxia is an autosomal recessive inherited neurodegenerative disease related to the reduced production of the mitochondrial protein frataxin. The clinical symptoms in Friedreich Ataxia include the in-coordination of limb movements, impairment of position and vibration, dysarthria, nystagmus, diminished or absent tendon reflexes, scoliosis etc. The onset of Friedreich's ataxia is early in life and almost always present before the age of 20 years. It is estimated that more than 95% of patients are wheelchair bound by the age of 45 years and due

to progressive multipathological effects the life span of patients is approximately 30–40 years with the most frequently reported causes of death being related to diabetes mellitus and cardiomyopathy (8,9,13,23).

Several factors are thought to be involved which can lead to variations in the progression of neurodegeneration, cardiomyopathy and other toxic side effects observed among Friedreich Ataxia patients. A major factor is thought to be related to the production levels of the protein frataxin and also several other factors influencing the rate of accumulation and toxicity of iron in mitochondria (8,9,13).

The localisation of focal deposited iron in the brain has recently been identified by MRI T2 and T2* relaxation times in many neurodegenerative and other diseases in addition to Friedreich's ataxia, such as Parkinson's and Alzheimer's diseases and Hallevorden-Spatz syndrome (7,13). In contrast, no iron accumulation in the brain or related toxic side effects involving the nervous system is observed in iron loaded thalassaemia or hereditary haemochromatosis patients (18,20).

Several other acquired and hereditary conditions with abnormal iron distribution leading to body iron imbalance can also lead to specific tissue iron localisation and anaemia. In these conditions different mechanisms and pathways of iron metabolism apply. For example in the anaemia of chronic disease, which is observed in many chronic inflammatory and other conditions such as rheumatoid arthritis, chronic kidney disease and cancer, iron is mostly transferred and stored in the cytoplasm of reticuloendothelial macrophages. The abnormal iron distribution in these conditions causes a reduced iron transfer from the reticuloendothelial macrophages into plasma and subsequently a reduction of iron availability to the bone marrow, leading to a reduction in haemoglobin production and consequently anaemia. Oral or intravenous iron and erythropoietin are used for increasing the production of haemoglobin and the general treatment of the anaemia of chronic disease.

A similar mechanism of plasma iron reduction is thought to operate in the hypoferraemia of infectious diseases. This host defence mechanism appears to reduce transferrin bound iron and iron bioavailability to microbes thus restricting their growth. This mechanism is particularly important for iron loaded patients who are more susceptible to siderophilic bacteria infections and have increased incidence of morbidity and mortality associated with infections (24–26). In this context, monitoring of serum iron and transferrin iron saturation and also use of pharmacological modulation of iron metabolism, as well as chelation therapy may be potential strategies for controlling and treating related infections (24–26).

5. MONITORING THE EXCESS IRON LEVELS IN IRON OVERLOAD

Several diagnostic techniques are used for monitoring the levels of excess iron, as well as the progress of chelation therapy in iron loaded thalassaemia and other transfused refractory anaemia patients. Most of these techniques are also generally used for the monitoring of patients with other diseases of iron metabolic imbalance, including the effects of phlebotomy in the treatment of iron overload in idiopathic haemochromatosis patients.

The most common and general methods for estimating body iron status are serum ferritin, serum iron, transferrin iron saturation and haemoglobin levels. Several other non routinely used methods include non-transferrin-bound iron (NTBI) and urinary iron excretion in response to chelating drugs such as DF and L1 (Figure 1) (13, 27,28).

Under normal conditions, the physiological non-toxic levels of iron are usually characterised by the normal serum ferritin values (male 40–340 µg/l and female 14–150 µg/l), transferrin saturation (25–35 %), serum iron 10–40 µmol/L and haemoglobin 11.5–17.5 g/dL. Similarly, the non-toxic liver iron concentration levels, equivalent to not more than 7 mg iron per g liver dry weight are considered as the maximum normal physiological levels in the assessment of liver biopsies. Higher levels of iron in the liver have been classified to various grades of liver iron concentration which correspond to different levels of iron overload with the higher grades to be associated with liver cirrhosis and fibrosis (10–12, 29–32).

The concentration of excess iron in the heart was until recently impossible to identify by using biopsies or other methods. However, the introduction of the new non-invasive diagnostic methods of MRI T2 and T2* relaxation time techniques, have successfully been used for estimating the level of iron loading not only in the heart but also the liver and other organs (Figure 2 and 3) (10–12,30).

Furthermore the introduction of the MRI T2 and T2* relaxation time techniques in combination with histochemistry of mainly liver biopsy samples and electron microscopy has contributed in the evaluation of the mechanisms of iron deposition and toxicity in various organs of iron loaded patients and also the design of targeted therapies for the removal of excess iron (13,28–32).

Estimation of the iron concentration of liver biopsy samples, usually reflect the total body iron store levels. In addition, the use of histochemical techniques of the liver biopsy samples can lead to the identification of the non-haem iron concentration and

the level, as well as the pattern of iron deposition in hepatocytes and Kupffer cells (30,31,33). An important drawback of the iron estimation of this method is that the distribution and concentration of iron in liver biopsy samples is not uniform (Figure 4). Furthermore, this method does not reflect the iron load or distribution in other organs such as the heart (Figure 2–4) (34–37).

The newly developed non-invasive techniques of iron estimation using the MRI T2 and T2* relaxation times are also becoming very useful tools for assessing differential organ iron deposition and in particular cardiac iron load levels, which are considered critical in the prognosis of thalassaemia and other transfused iron loaded patients (Figure 2) (7,13,29).

The MRI and all other methods described above can be used periodically for assessing the prognosis of the patients in relation to iron toxicity, which is directly related to their total body or individual organ iron load and organ function. Similarly, the same methods can also be used for the adjustment of the dose protocols during iron chelation therapy in transfusional iron overload or the phlebotomy protocols in idiopathic haemochromatosis patients.

Regular clinical and biochemical assessment is generally used in addition to monitoring iron levels, in order to evaluate organ function and the extent of damage caused by iron overload and other complications of the underlying diseases in each category of iron loaded patients (Table 1). In thalassaemia patients monitoring of the frequency of the iron loading process and the progress of the chelation therapy is usually carried out by estimating serum ferritin levels every three months, while liver iron and cardiac iron concentration estimation can be carried out annually by using the MRI T2 and T2* relaxation time techniques (Table 1) (13,29). In the absence of MRI facilities, the estimations of liver iron can also be carried out using liver biopsies samples, which appear in most cases to correlate well with serum ferritin levels.

There are different classifications of iron overload and toxicity in relation to different organs and especially the heart and the liver. Regarding cardiac iron levels, it was observed that patients with cardiac MRI T2* relaxation times lower than about 8 ms are considered to be in the heavy haemosiderosis range and to be in danger of cardiac failure. Moderate cardiac haemosiderosis is considered for patients with cardiac MRI T2* relaxation times in the range of about 8–12 ms, mild haemosiderosis in the range of about 12–20 ms and above 20 ms to have normal cardiac iron levels (11,12,29,34–38). Similar classifications have been described for liver iron overload where MRI T2* relaxation times have been compared to the iron

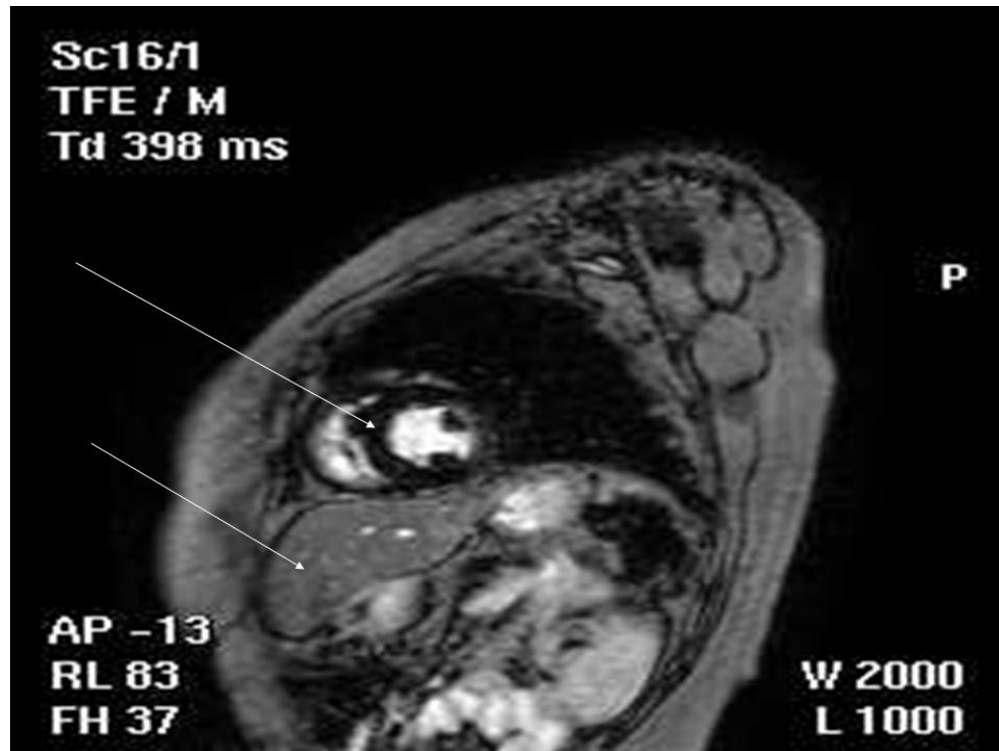


Figure 2. MRI picture and T2* relaxation time estimation of an iron loaded thalassaemia patient, showing differential iron loading of the heart and liver. Heavy haemosiderosis of the heart (T2* = 6.32 ms (normal T2* = >19 ms)) and normal T2* of the liver (T2* = 19.2 ms). The top arrow shows the excess iron deposition in the interventricular septum of the heart of the patient, which is shown with low signal intensity (dark). The bottom arrow shows the liver of the patient with no excess iron deposition (normal). Adapted from reference 18.

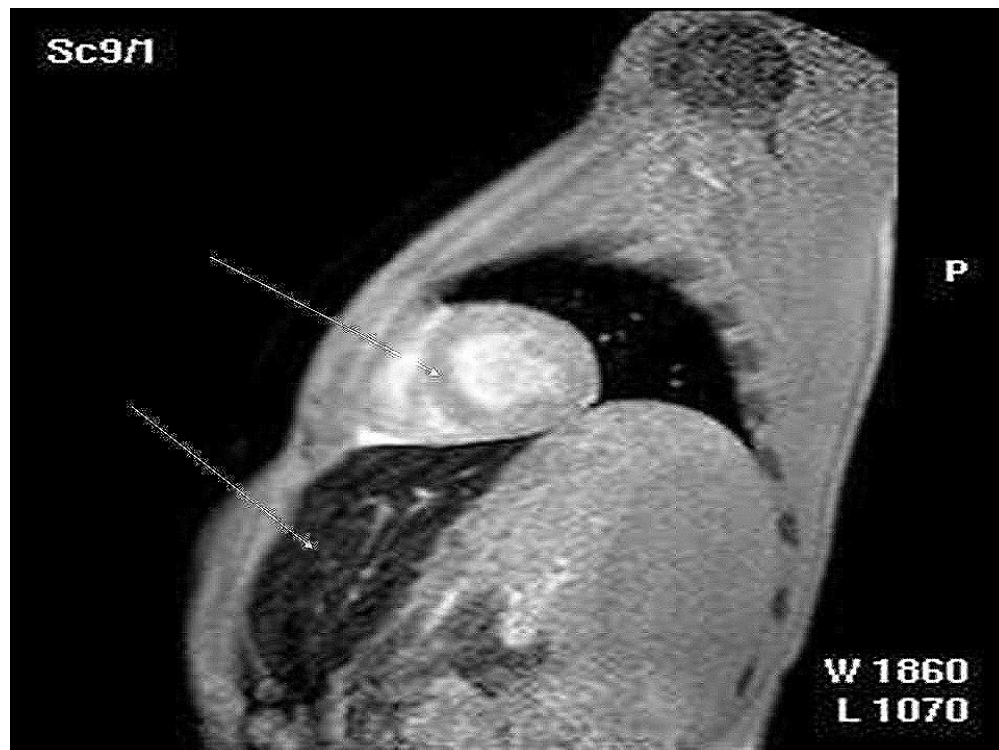


Figure 3. Differential distribution of iron deposits in the heart and liver of a 29-year-old male thalassaemia patient with a serum ferritin level of 905 µg/L. Short axis view of heart (top arrow) and liver (bottom arrow) indicating a mosaic pattern of excess liver iron deposition with different low signal intensity iron in the range of T2* 4.1 ms and of T2* 3.4 ms. Cardiac iron load at the interventricular septum was estimated at T2* = 33 ms (normal). Adapted from reference 12.

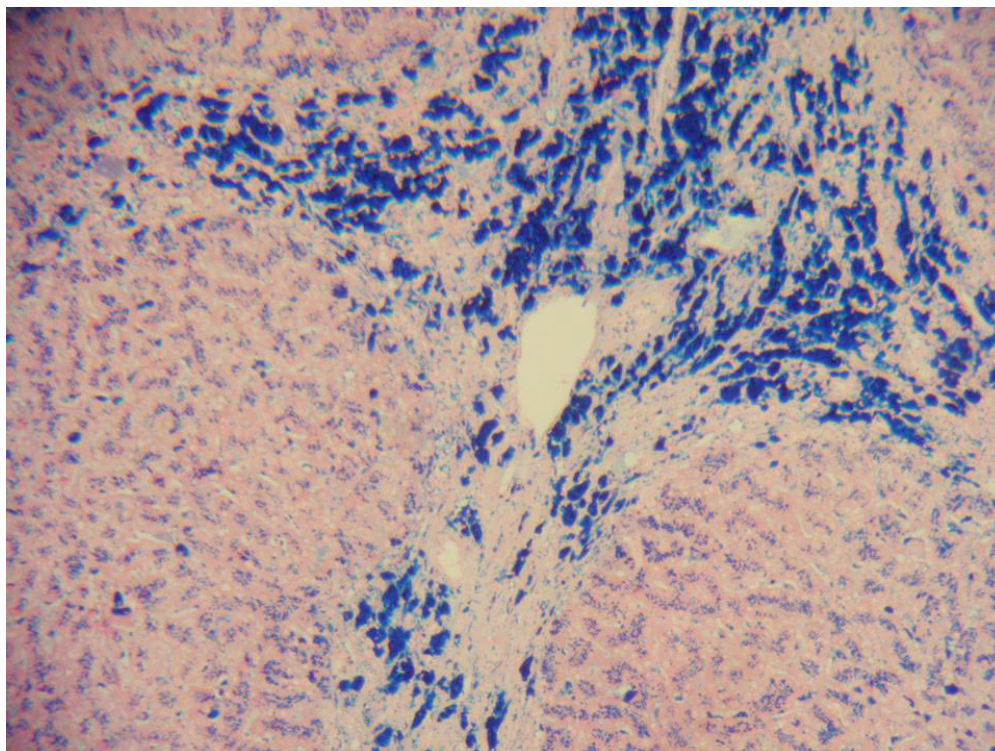


Figure 4. Non homogeneous distribution of excess iron in the liver of a thalassaemia patient. Liver biopsy photograph (×20) of a 29-year old, 55 kg male thalassaemia patient. Liver section showing non uniform iron deposition stained with Pearl's Prussian blue. There are haemosiderin deposits in hepatocytes and Kupfer cells and especially within bile ducts. Four months before the splenectomy the patient had an MRI T2* (ms) of heart 4.1, liver 0.0, spleen 2.9, and serum ferritin of 3850 µg/L. Adapted from reference 39.

Table 1. Clinical and biochemical monitoring routinely used in thalassaemia major patients

Monitoring at monthly intervals	Serum ferritin for patients with normal iron stores. Serum ferritin and zinc concentration in iron loaded patients. (Every 3–4 months). Full blood count. (Every 1–2 weeks for patients treated with L1).
Monitoring at half yearly intervals	Liver and kidney function tests. Urate, cholesterol, triglycerides, Ca, PO4. Fasting glucose test. Prothrombin time.
Monitoring at yearly intervals	MRI T2 and T2* of the heart and liver. Blood glucose tolerance test. Screening for hepatitis B antibodies, hepatitis C and HIV. Bone densitometry. Chest X-ray. Endocrine (T3, T4, TSH, PTH). Ophthalmology, ear, nose, throat and echo of the abdomen examinations. Cardiac: Physical examination, echocardiography (ECG), holter, stress test, muga scan. Supporting therapy from a specialised team: Cardiologist, endocrinologist, haematologist, paediatrician, dietician, gynaecologist, psychologist.

content of liver biopsy samples. In this context, MRI T2* relaxation times lower than 1.4 ms are estimated to contain more than 10 mg of iron per g dry weight of the biopsy sample and considered to be in the severe hepatic haemosiderosis range. Moderate hepatic haemosiderosis is considered for MRI T2* relaxation times in the range 1.4–2.7 ms, corresponding to 5–10 mg/g dry weight, mild in the range of 2.7–6.3 ms corresponding to 2–5 mg/g dry weight and normal

hepatic iron for higher than 6.3 ms corresponding to less than 2 mg/g dry weight (11,12,29,34–38).

In this context, serum ferritin levels greater than 2500 µg/l, transferrin iron saturation of 100% and liver iron concentration greater than 7 mg iron per g liver dry weight suggest the presence of toxic levels of iron in the tissues and the need for the use of higher doses and more intensive chelation therapy. It is also

considered that above these iron levels, the damage to organs may in the long term become irreversible. The improvement in cardiac and liver iron estimations using MRI T2 and T2* are progressively becoming the most useful and reliable techniques for the regular assessment of transfused iron loaded and idiopathic haemochromatosis patients (10–13,29).

Despite the overall improvements using the above established methods of monitoring iron levels, there can also be limitations in the prediction of either the total body iron load or the extent of iron toxicity. These limitations are observed because of many variables such as differences in the iron assessment and detection methods, in differences in the total body and organ iron distribution of stored iron, in the specificity of iron pool and organ iron removal effects of different chelating drugs or chelation therapy protocols of transfusional iron loaded patients etc. Similar limitations are also observed in the case of idiopathic haemochromatosis patients in relation to the assessment of phlebotomy therapy protocols. A number of other factors can also influence the iron metabolic processes or the estimation of the iron load levels as a result of individual variations such as dietary habits, infection, inflammation, rate of iron absorption and excretion, transfusion frequency, erythropoietic activity, bleeding etc.

6. IDENTIFICATION OF THE IRON METABOLIC PATHWAYS INVOLVED IN IRON OVERLOAD TOXICITY

The identification and characterisation of the iron overload toxicity pathways are essential for the design of therapeutic strategies for the treatment of related diseases.

The combination of the assessment of iron overload using iron biomarkers, the histopathology of liver and spleen biopsy samples, the use of electron microscopy and especially the recent introduction of the MRI T2 and T2* relaxation time techniques has led to a reassessment of previous theories and also a new understanding of iron metabolic pathways related to iron overload toxicity (Figure 2–5) (10–12, 28–32).

Under normal conditions iron is thought to be regulated through the physiological pathways of iron absorption, excretion and utilisation, including iron reutilisation from the breakdown of effete red blood cells. These are considered to be the major pathways of iron metabolism for the uniform distribution of iron in the various organs and for maintaining body iron balance (1, 2, 18, 28, 33).

Many changes are observed in iron overloading conditions in relation to the iron metabolic pathways, deposition of iron in the various organs and

also the body iron balance in general. For example, in hereditary haemochromatosis the storage of excess iron from increased gastrointestinal iron absorption is primarily deposited in the parenchymal cells of the liver. However, a different metabolic pathway is observed in transfusional iron loading conditions, where the storage of excess iron from the breakdown of effete red blood cells following repeated transfusions is mostly deposited initially in the Kupffer cells of the liver and spleen and then transferred and deposited in the parenchymal cells (1, 2, 18, 28, 33).

It was considered until recently and before the introduction of the MRI T2 and T2* relaxation time that both in hereditary haemochromatosis and transfusional iron overload in thalassaemia, iron was uniformly distributed in the various organs and that serum ferritin and liver iron reflected the levels of the iron stores in the body.

However, many recent clinical findings and iron load estimations using MRI T2 and T2* relaxation time in iron loaded thalassaemia patients suggested that serum ferritin is in most cases only related to liver iron stores but not to spleen, heart and pancreatic iron load (Figure 2) (34–37). Furthermore, it was also observed using MRI that in some cases of thalassaemia patients the liver was overloaded with iron but the heart had normal iron store levels (Figure 3). In contrast, in some other thalassaemia patient cases the reverse was true ie the heart was overloaded with iron but the liver had normal iron concentration levels (Figure 2) (18). It should be noted that this last finding using MRI provides an explanation for several fatal cases of thalassaemia patients due to congestive cardiac failure observed in the past, despite very low serum ferritin and liver iron concentration (Figure 2 and 5). Overall, it appears from studies using MRI T2 and T2* relaxation times and other techniques that serum ferritin levels can be misleading with regards to cardiac iron levels and this may increase the risk of cardiomyopathy in thalassaemia and other transfused patients (Figure 2) (34, 35, 37,38).

Further studies using MRI T2 and T2* relaxation time, histopathology and other techniques suggested variability in the iron accumulation and deposition processes in other organs. In particular, the importance of the role of the spleen as a major storage organ for iron in addition to the liver was also recently highlighted (39). In this context it appears that the spleen in some thalassaemia patients with splenomegaly has sometimes equal iron storage capacity to that of the liver. Furthermore, the spleen can exert a great influence on the ferrikinetics and toxicity of iron overload and also the overall iron removal treatment of thalassaemia patients (39). In this context, not only an increase in haemoglobin was observed following splenectomy in thalassaemia patients but also a

substantial increase in serum ferritin, thus providing further evidence that serum ferritin is not related to total body iron load but mostly to the concentration of stored iron in the liver (39). Another observation following splenectomy in thalassaemia patients is that excess iron from the repeated transfusions may not only increase liver iron deposition but can be also be diverted to the heart causing excess myocardial iron loading and cardiomyopathy (40).

The recent MRI and other related findings suggested that in general serum ferritin levels and liver iron concentration estimations are misleading regarding cardiac and other organ iron load levels, as well as total body iron load levels in thalassaemia patients (36, 37).

Furthermore, recent MRI T2 and T2* relaxation time findings appear to suggest that in many cases of iron loaded thalassaemia patients the deposition of iron in the liver, spleen and heart is not only differently distributed between these organs but is also not uniformly distributed within each organ (Figure 3) (12). In this context, it appears that there is sometimes mosaic iron distribution of dense and light iron deposits in the liver and heart and this is particularly evident during the process of the removal of excess iron and the normalisation of the iron stores in thalassaemia patients treated with the L1/DF International Committee on Chelation (ICOC) combination protocol (Figure 3) (12). The non uniform iron distribution of iron deposits can also be observed in the liver and spleen biopsy specimens of thalassaemia patients, thus providing a further explanation for the high level of error of liver biopsies for estimating iron load and total body iron store levels (Figure 4) (37,39).

7. THE MONITORING OF THE EFFICACY OF CHELATING DRUGS IN IRON OVERLOAD

The main worldwide use of iron chelating drugs and primarily of DF, L1 and deferasirox (DFRA) is the removal of excess iron in thalassaemia and other transfusional iron loading conditions, where excess iron leads to progressive multi-organ damage and associated increase in morbidity and mortality in the affected patients (Figure 1) (16–18). In addition to transfusional iron loaded conditions, iron chelation therapy could also be used for many of the abnormalities of iron metabolism including conditions related to focal or localised excess iron deposition and toxicity (7, 16–18, 26). In this context, the general mode of action of chelating drugs in all these conditions is the removal, donation or exchange of iron which can bypass, supersede or enhance physiological mechanisms and can lead to the correction of the iron abnormality (18). Such chelator interventions may restore iron metabolic balance and eliminate or minimise the associated iron toxicity.

The clinical mode of action, efficacy and toxicity profiles of the three main chelating drugs DF, L1, DFRA and also of other iron chelators in iron overload and other conditions are directly related to their iron binding, physicochemical, pharmacological, toxicological and other properties (Figure 1, Table 2) (5,18,26).

The prognosis of thalassaemia patients and of other categories of regularly transfused patients is different between the clinical groups, hospital units and also countries and generally depends on the iron load but also many other factors related to their underline medical condition (13, 16, 17). In the absence of chelation therapy regularly transfused thalassaemia patients die by the age of 20 years mainly from congestive cardiac failure caused by cardiac iron overload toxicity, whereas treatment with subcutaneous DF can increase mean survival to about 35 years (Figure 5) (16, 17, 41). However, the recent introduction of specific chelation therapy protocols of L1 and L1/DF combinations appear to increase life expectancy and reduce the mortality of iron loaded thalassaemia patients to levels approaching those of normal individuals (13,42).

The worldwide availability and clinical application of the three chelating drugs DF, L1 and DFRA depends on many factors including mainly the efficacy, tolerance, site of action, toxicity profile and cost (Table 2) (7,18,43). All these differences variably affect the general morbidity and mortality rate of thalassaemia and other transfusional iron loaded categories of patients both in developed and developing countries (13, 42, 43). In particular, the majority of the thalassaemia patients and especially those living in the developing countries are left to die without treatment because of the lack of financial resources and inability to pay the high cost of the transfusions and chelating drugs (13–15).

Regarding clinical aspects, there are several variables which can affect the efficacy of chelation treatment in iron loaded patients not only in relation to the levels of iron overload and organ distribution but also due to differential response and other pharmacological property effects of each of the chelating drugs. The variable responses include individual profile differences in the absorption, distribution, metabolism, elimination and toxicity (ADMET) of the chelating drugs (44,45). For example, orally administered L1 causes an increase of iron excretion exclusively in the urine, whereas orally administered DFRA an increase of iron excretion exclusively in the faeces and subcutaneously administered DF an increase in iron excretion mostly in the urine but also the faeces (Table 2) (18). In this context, the efficacy and toxicity of chelating drugs is monitored regularly in each patient by following the level of iron excretion, the iron biomarkers for

Table 2. General properties and mode of action of the iron chelating drugs

Recommended doses for the chelating drugs in thalassaemia patients	Subcutaneous or intravenous DF: 40–60 mg/kg/day. Oral L1: 75–100 mg/kg/day. Oral DFRA: 20–40 mg/kg/day.
Iron loaded patient compliance with chelating drugs	Low compliance with subcutaneous DF in comparison to oral L1 and oral DFRA. Better compliance with DF in combination therapies.
Combination chelation therapy	L1, DF and DFRA combinations are more effective in iron excretion than monotherapies. The ICOC combination protocol of L1 (75–100 mg/kg/day) and DF (40–60 mg/kg at least 3 days/week) causes normalisation of the iron stores in thalassaemia patients. The level of iron excretion and the rate of normalisation depend mainly on the drug doses and iron load of the patients.
Increase in iron excretion and route of elimination in iron loaded patients	L1: Increase in urinary iron excretion. DFRA: Increase in faecal iron excretion. DF: Increase in both urinary and faecal iron excretion.
Effect of chelating drugs on iron absorption	Increase of iron absorption by the lipophilic chelating drugs maltol and DFRA. Decrease of iron absorption by the hydrophilic chelating drugs DF and L1.
Differential iron removal from various organs of iron loaded patients	L1: Preferential iron removal from the heart and also liver. DFRA: Iron removal mainly from the liver. DF: Iron removal from the liver and also less so from the heart. (Efficacy in iron removal is related to the dose of the chelating drugs).
Iron removal from diferric transferrin in iron loaded patients	Transferrin iron removal of about 40% at L1 serum concentrations of > 0.1. mM, but no iron removal by DF or DFRA. (Transferrin is 25–30 % saturated with iron in normal individuals and usually 100 % in thalassaemia patients)
Iron redistribution in diseases of iron metabolism by chelating drugs	L1 and to a lesser extent DF can cause iron redistribution from the reticuloendothelial system to the erythron in the anaemic rheumatoid arthritis patients. Enterohepatic circulation by DFRA and metabolites.
Iron mobilisation and excretion of chelator metabolite iron complexes	Several DF metabolites have iron chelation potential and increase iron excretion. Both the L1 glucuronide and the DFRA glucuronide metabolites have no iron chelation properties.
Chelating drug synergism with reducing agents	Ascorbate act synergistically with DF but not with L1 and DFRA for increasing iron excretion.
General toxicity of the iron chelating drugs	DF: Ocular and auditory toxicity, allergic reactions at the sites of the injections, Yersinia enterocolitica infections. L1: Low incidence of agranulocytosis, neutropenia, musculoskeletal complications, gastric intolerance. DFRA: Renal, liver and bone marrow failure including agranulocytosis, as well as renal toxicity, skin rashes, gastric intolerance. Increase in serum creatinine.

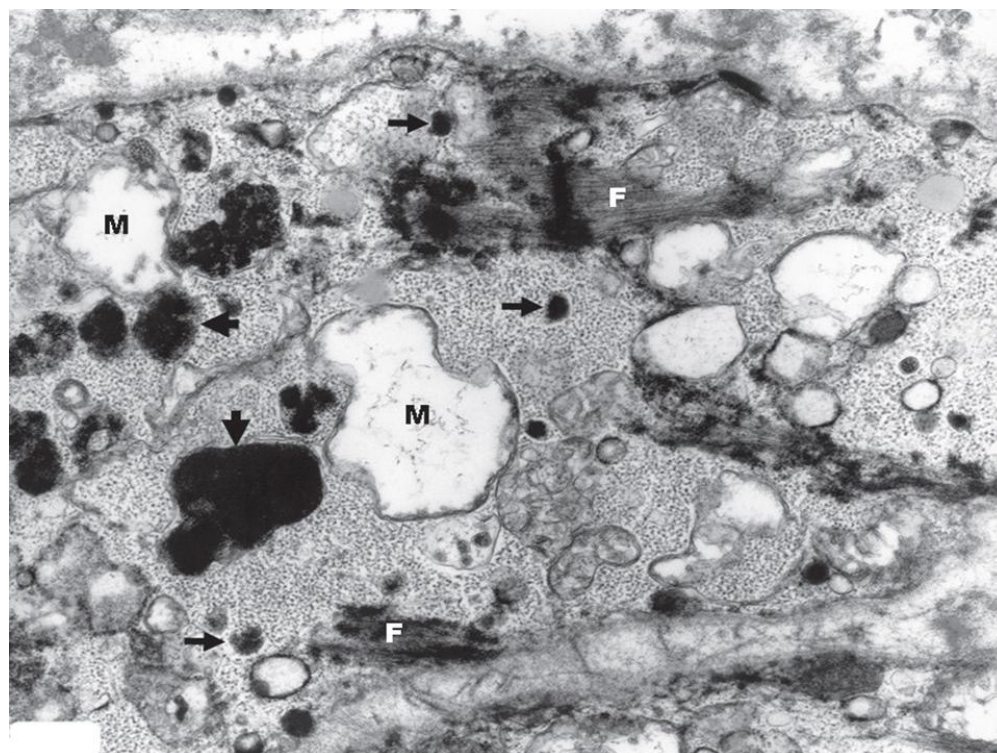


Figure 5. Electron micrograph of a cardiac biopsy of the thalassaemia patient at X30000 magnification. It shows a myocyte with extensive iron deposition mostly in primary (arrows) and secondary (arrowheads) lysosomes, loss of myofilaments (F) and swollen/damaged mitochondria (M). Adapted from reference 38.

the whole body as well as individual organ iron load status and also the possibility of other complications in relation to the underlying disease. In each patient case, individual protocols can be designed for optimal chelation therapy based on the different properties of the chelating drugs and the response to the chelation therapy (46).

The optimum doses of chelation therapy for each of the drugs is different and general recommended doses in thalassaemia and other transfusional iron loaded conditions usually vary from 40–60 mg/kg/day for subcutaneous DF, 75–100 mg/kg/day for oral L1 and 20–40 mg/kg/day for oral DFRA (Table 2) (43–46). Selected therapeutic protocols can be designed and used in various categories of iron loaded patients and in targeted therapies which can result in the application of different range of doses from intensive chelation, eg combinations of DF (40–60 mg/kg/day) and L1 (75–100 mg/kg/day) in heavily transfused iron loaded patients, to intermittent withdrawal of chelation therapy in thalassaemia patients who have achieved normal iron stores and for whom further chelation therapy may cause iron deficiency and other complications (43,46,47).

The selection of the chelation therapy protocols for iron loaded patients in each clinic or country is not uniform and depends not only on the iron load of patients but also on clinical, marketing and other factors, and especially the cost of treatment (48). In general, there is a lack of consensus in the ultimate aim of the chelation protocols for optimal therapy in transfusional iron loaded patients (46,48). Similarly, there is no consensus in the evaluation criteria including iron biomarkers and risk/benefit assessment for the use of each of the chelating drugs in personalised medicine (46,48). In developing countries where most thalassaemia patients live, the main issue with regards to iron chelation therapy is the cost of the chelating drugs and their availability and not necessarily the risk/benefit chelating drug assessment issues. These restrictions do not only apply to chelating drugs but also to most other orphan drugs, which are related to many other orphan diseases like thalassaemia (46,48).

The monitoring of efficacy of different chelation therapy protocols on gross body as well as individual organ iron load levels has been recently achieved following the introduction of the MRI T2 and T2* relaxation time techniques. These methods have increased the prospects of the introduction of personalised medicine by increasing our understanding of iron metabolic and iron chelation pathways, especially in relation to iron removal in transfusional iron overload. Similarly, they have improved chelating drug targeting therapies for iron deposits in individual organs which are the cause of toxic side effects and of increased morbidity and mortality in iron loaded

and other categories of patients (26,36,46, 49,50). In particular, following the use of these new diagnostic methods in combination with serum ferritin monitoring, it was possible to introduce chelation therapy protocols of L1 or the L1/DF combination, such as the ICOC protocol, which can achieve the complete elimination of iron overload from the heart and liver of thalassaemia patients (51–56).

The clearance rate of excess iron from the heart and liver in most categories of iron loaded thalassaemia patients is variable and in general it can be completed in a period ranging between 0.5 to 1.5 years using the ICOC protocol of L1 (75–100 mg/kg/day) and DF (40–60 mg/kg/day at least 3 days per week). Usually the clearance rate of excess iron from these organs is faster when using higher overall doses of L1 and DF and in patients who are not heavily iron loaded and are not receiving high number of transfusions (51–56). The removal of excess iron deposits by L1 and the L1/DF combination in thalassaemia patients is accompanied with the improvement of organ function, such as the elevation of the left ventricular ejection fraction (LVEF) and other cardiac and endocrinological improvements (38,52,53).

The removal of excess iron from the heart by DFRA even after long term treatments seems to be much less effective and is also very slow, with no improvement in LVEF, especially in heavily iron loaded thalassaemia patients. For example, in a single centre study even after 5 years of treatment with DFRA (30–40 mg/kg/day) excess amounts of iron appear to still remain in the heart of thalassaemia patients and there was no improvement in LVEF (57,58).

Excess iron removal from the liver by DFRA seems not to be as effective even at high doses and is also not equivalent to that resulting from DF, L1 and their combination when the latter two chelating drugs are administered at effective doses (43,51,52,59). The most effective and rapid clearance of excess liver iron which can also be used for intensive chelation therapy in heavily iron loaded patients is the administration of intravenous DF in combination with oral L1.

Completely different chelation therapy protocols are used in thalassaemia patients following the achievement of normal iron stores and the clearance of excess iron from the liver and heart, which are generally characterised and confirmed by normal levels of MRI T2 and T2* relaxation times and serum ferritin. In these cases, the normal iron store levels can usually be maintained by using much lower overall chelation doses and in particular L1 monotherapy (51–54). The monitoring of serum ferritin levels at monthly intervals is also necessary in this category of patients for avoiding chelating drug overdose toxicity as previously shown in some cases of over-chelated

thalassaemia patients (Table 2) (47). Similarly, lower L1 doses and periodical withdraw of chelation therapy may be necessary in some patients in order to avoid over-chelation of iron and associated toxicity (47,51–54). The use of DF and DFRA is not generally recommended for thalassaemia patients with normal range levels of iron stores or with serum ferritin less than 500 µg/l due to toxicity complications (13,43).

The continuous and thorough monitoring of the total body as well as individual organ iron store levels allows for the design of targeted chelation therapies and also the design of personalised medicine chelation protocols for the complete elimination of iron overload and related toxicity in thalassaemia and other categories of iron loaded patients.

8. THE EFFECTS OF CHELATION THERAPY IN NON IRON OVERLOAD DISEASES

The possibility of using chelating drugs for correcting iron metabolic abnormalities or for influencing iron related pathways in many diseases in addition to iron overload was suggested and tested in a number of *in vitro* and *in vivo* studies (7,60–65). Clinical trials involving different categories of patients with iron metabolic imbalance, which are not related to iron overload have also been carried out using different chelation therapy protocols. In most of these patient categories, iron imbalance or toxicity is manifested despite that iron is deposited or distributed in the body in a different way to that observed in normal individuals or in patients with iron loaded diseases.

The clinical studies in the categories of patients with normal iron stores were mostly carried out using L1 because of its high safety profile in comparison to DF and DFRA (5). In one such study the effect of up to 2x2 g/day of L1 administration for a week was carried out for testing the hypothesis of restoring haemoglobin levels in the anaemia of chronic disease. The investigation involved a group of anaemic rheumatoid arthritis patients including some patients not responding to erythropoietin treatment. A substantial increase in haemoglobin levels was observed following the treatment with L1 in this cohort of patients (61,62). The anticipated mechanism operating in patients with the anaemia of chronic disease was thought to involve an initial stage of mobilisation of stored iron by L1 from the reticuloendothelial macrophages and other sites, the donation of iron to unsaturated transferrin resulting in an increase in transferrin iron saturation, and lastly the transfer of increased iron in the form of transferrin iron to bone marrow and other erythropoietic tissues causing an overall increase in the production of haemoglobin (7,61–65).

Removal and utilization of focal iron deposits involving different organs or tissues was observed in

several other iron metabolic imbalance diseases. For example, the removal of iron from focal iron deposits in the brain has been shown using L1 in a number of clinical trials involving Friedreich's ataxia patients. In one such study, Friedreich's ataxia patients were treated with 20–30 mg/kg/day of L1 for 6 months resulting in a substantial reduction of the stored excess iron in the brain which was diagnosed using MRI T2* relaxation time. The reduction of the toxic iron deposits in the brain was accompanied by clinical improvements including reduction in ataxic gait and neuropathy (66). Neurological and heart function benefits were also identified in further L1 trials in Friedreich's ataxia and similar other categories of patients with neurodegenerative diseases (66–68).

Focal or labile iron deposits have been implicated in tissue damage of many other diseases, which were also targeted by chelation therapy resulting in the prevention or minimisation of iron toxicity (5). For example, improved therapeutic outcomes were observed in clinical studies involving about fifty kidney disease patients using L1 at doses of 50–75 mg/kg/day for up to 9 months (69). In this category of patients L1 was shown to improve kidney function and to cause a decrease in proteinuria, with no serious toxic side effects (69).

The use of iron chelating drugs in many other conditions involving different iron metabolic pathways such as infections, inflammation, drug toxicity and cytotoxic therapies, the detoxification of other metals as well as many other conditions involving proteins and pathways of iron metabolism is currently under investigation (18,25,26,43,70,71). Similarly, the application of L1 as a pharmaceutical antioxidant in many diseases of free radical pathology and ageing is also under evaluation (5).

9. CONCLUSION AND FUTURE PROSPECTS

Iron plays a major role in many physiological processes including the normal growth and development of humans. Iron can also play a significant role in many diseases related to iron metabolism and its control by chelating drugs can lead to effective treatments. Identification of the factors causing the iron metabolic abnormality and diagnosis of the level of toxicity can lead to improved therapeutic approaches.

The use of new, non invasive iron diagnostic methods such as MRI T2 and T2* relaxation times and also of other methods of iron estimation and organ iron deposition and storage has increased our understanding of iron metabolic and toxicity pathways involved in diseases of iron overload. In this context, the contributory effects of many environmental, iatrogenic and metabolic factors related to abnormal iron deposition, has been identified and linked to iron

toxicity pathways leading to clinical complications (Figure 2–5) (5–7,10–13,56).

The development of new iron metabolism biomarkers such as determination of hepcidin levels, as well as new and more efficient MRI and other diagnostic methods could increase further our understanding of the mechanisms involved in many other diseases related to abnormalities of iron metabolism (6,7,56, 60). Further improvements in iron diagnostic methods, especially at the molecular level could also increase the prospects of more accurate and effective targeted therapies involving several other diseases of iron metabolic imbalance and toxicity in the future, many of which have not yet been fully characterised.

The introduction of targeted chelation therapies are expected to lead to more effective and less toxic personalised optimal treatments. This approach is feasible because of differences in the physicochemical, pharmacological and other properties of the chelating drugs and other chelators under development for clinical use (7,13,18,46). The introduction of targeted chelation therapies have so far increased the prospects of better treatments for iron loaded patients, as well as many other groups of patients with focal iron deposition which is known to lead to tissue damage and other toxic side effects (5,46, 66–69). This approach can be developed further and include improved treatments for many other disease categories related to abnormal iron metabolic pathways including iron deficiency, cancer, free radical pathology and ageing (5,7,66–69).

New chelation therapy protocols, such as the ICOC protocol were introduced for the effective removal of iron from the heart and also the complete clearance of excess iron from other organs of thalassaemia patients leading to an overall decrease in morbidity and mortality in this category of patients (12,41,42, 51–54). Similarly, specific chelation therapy protocols were introduced for Friedreich's ataxia and other categories of patients with focal iron deposition and toxicity, which also led to clinical improvements (66–68).

Future trends in the application of chelating drugs for controlling iron metabolic imbalance is also highlighted from the recent introduction of iron maltol for the treatment of iron deficiency in inflammatory bowel disease (Figure 1) (72–74). The treatment of cardiac iron overload in aceruloplasminaemia, juvenile haemochromatosis and other conditions using L1 and DF combination therapy are further examples of targeted therapies (70,75). Similarly, the development of intranasal DF for crossing the blood brain barrier with possible clinical use in neurodegenerative and other diseases is a different form of targeted therapy, which is related to improved formulation and administration

of chelating drugs (76,77). The use of chelating drugs for specific therapeutic applications targeting cancer, renal, infectious and other diseases is also currently in progress (5,69,78).

Targeted chelation therapies in cancer are in an advance stage of development and new investigational anticancer chelating drugs are in the pipeline (79–81). The targets for cancer therapeutics related to iron metabolism are many and different and are not only related to the chelating drugs used in iron overload. In some cases the metal complexes of chelating drugs are used for targeted anticancer activity (82–84). Such anticancer activity may also be related to specific mechanisms, pathways or molecular targets or several of these components (82–84). The possibility of using chelating drug combinations as well as combinations with established anticancer drugs instead of monotherapies may improve anticancer targeting therapies in specific cancer types.

The identification of the characteristics of the iron pools at the molecular, sub-cellular, cellular, tissue and organ levels and the development of new diagnostic techniques has helped in the identification and characterisation of normal and abnormal iron metabolic pathways leading to new therapeutic approaches for a range of diseases related to iron metabolism (8–12, 85–88). The same methods have also been used in the monitoring of different therapeutic protocols and the effect of targeted chelation therapies for specific tissues and organs with encouraging results and overall improvements in the therapeutic outcomes and safety of iron loaded and other categories of patients.

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