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Review Article

Iron Toxicity and Hemopoietic Cell Transplantation: Time to Change the Paradigm

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Abstract. The issue of iron overload in hemopoietic cell transplantation has been first discussed in the field of transplantation for thalassemia. Thalassemia major is characterized by ineffective erythropoiesis and hemolysis leading to severe anemia. Patients require regular blood transfusion therefore they develop iron overload causing organ damage and hematopoietic cell transplantation (HCT) is a consolidated reliably curative option.

In this category of patients an important issue for transplant outcome is the iron burden before transplant and in the long-life post-transplant. Nevertheless today the concept of the impact of iron overload / toxicity on the outcome of HCT has been extended to other diseases characterized by periods of variable duration of transfusion dependence .

Recent preclinical data has shown how increased production of reactive oxygen species (ROS) resulting under iron overload condition, could impair the stem cells clonality capacity, proliferation and maturation. Also, microenvironment cells could be affected through this mechanism. For this reason, iron overload is becoming an important issue also in the engraftment period post-transplant.

The aim of this review is to update consolidated knowledge about the role of iron overload/iron toxicity in the HCT setting in non-malignant and in malignant diseases introducing the concept of exposition of free toxic iron forms and related cellular damage in the different stage of transplant.

Keywords: Hematopoietic stem cell transplantation; Iron overload; Thalassemia.

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Introduction. ß-thalassemias are a group of genetic hemoglobinopathy presenting different grading of ineffective erythropoiesis and hemolysis leading to anemia in the majority of patients. ß-Thalassemia Major (ß-TM) is the most common form of thalassemia. Patients affected by ß-TM require regular blood transfusion therefore they develop iron overload causing organ damage.[1] This condition is therefore defined as Transfusion Dependent Thalassemia (TDT).

More than 30 years have passed since the first successful Hematopoietic Cell Transplantation (HCT) in thalassemia. Since then, more than 3000 transplants have been performed worldwide with outstanding results.[2] Gene therapy has been now finally developed and promises to be a further improvement in thalassemia cure option.[3]

Firstly in this category of patients iron overload toxicity has emerged as an important issue for transplant outcome. Clinical results revealed how iron exceeded before HCT affects the outcome in TDT. Subsequently the role of iron overload / toxicity have been investigated in others transfusion dependent

diseases including myelodysplastic syndromes (MDS). It is well known that iron overload is deleterious for organs such as liver, heart and endocrine glands and, it has been postulated could also increases the risk of infections and Graft versus Host Disease (GvHD) early after HCT. High baseline Ferritin levels before HCT have been shown to negatively influence clinical outcome in diseases different from TDT.[4-6]

If solid clinical evidence has established the negative impact of high iron burden and related tissue damage on the outcome of HCT for TDT, recent preclinical data has shown how increased production of reactive oxygen species (ROS) resulting under iron overload condition, could impair the hemopoietic niche and therefore the stem cells clonality capacity, proliferation and maturation independently from baseline disease. Also, microenvironment cells could be affected through this mechanism. Moving from this hypothesis, in-vitro and animal model experimental studies started to understand if iron toxicity could be an issue also in the HCT engraftment period.[7]

The aim of this review is to update consolidated knowledge about the role of iron overload/iron toxicity in the HCT setting introducing the concept of exposition of free toxic iron forms and related cellular damage.

Recently Understood Mechanisms Support Iron Toxicity and Cell Damage. Iron is an essential element for the normal cellular life but when it exceeds the needs of physiological cellular processes, reactive oxygen species are produced. Increased ROS levels may have beneficial or toxic effects depending on their levels. In the case of imbalance in intracellular redox homeostasis, ROS levels overwhelm cellular antioxidant defenses, and an oxidative stress state is established. Numerous cellular functions are determined upon appropriate intracellular ROS levels, and are deregulated under oxidative stress conditions. These processes involve the activation of signaling pathways leading to alterations of cellular cycle, proliferation, differentiation, and eventually cell death.[7]

Iron in Phisiological and Pathological Conditions. Thanks to its biochemical feature, iron moves across different oxidation states (ranging from Fe2- to Fe6+; the two common forms involved in human biochemical reaction are Fe2+ and Fe3+), this flexibility makes iron suitable for a variety of normal biochemical reactions particularly involving electron transport and mitochondrion activities. To maintain a stable level of iron and a normal iron homeostasis, living organisms must be able to release stored iron during iron deficiency and store excess iron during iron sufficiency in an appropriate manner. Under normal condition iron circulates binding to transferrin and systemic iron balance is mainly maintained by the iron regulatory hormone hepcidin that binding to ferroportin (FPN) on the cell surface, regulates the iron cellular efflux. When iron overwhelms the transferrin's capacity to transport iron, non-transferrin bound iron (NTBI) appears in the circulation and together with its active biological component, labile plasma iron (LPI), is able to enter the cell through canonical routes but also through alternative channels participating to increase the labile cell iron (LCI) pool **(Table 1).** LCI is the intracellular free iron form that contributes to the mitochondrial life, leading to hemoglobin production,[8] energy production throughout the Krebs cycle and iron sulfuric group formation that are fundamental for the DNA

Table 1. Free iron forms types and property.

Acronym	Meaning	Characteristic	Chemical composition	Redox active	Ability to enter cell by a transferrin independent way	Chetable
NTBI	non-transferrin bound iron	Circulating iron not bound to transferrin, ferritin or heme.	Heterogeneous. Several circulating isoforms that is FE(III) bound to albumin, citrate and potentially to acetate, malate and phosphate.	**No**	**Yes**	**Yes**
LPI	labile plasma iron	Fraction of NTBI that is redox active, potentially toxic. Also defined as extracellular counterpart of LCI	Heterogeneous. Components are the same of NTBI,	Yes	Yes	Yes
LCI	labile cellular iron	Fraction of the cellular iron pool that promotes ROS formation when it raises to "relatively high" levels*	It is comprised of complexes of iron with nucleotides, gluthathione and carboxylates	Yes	Na	Yes
eLPI	enhanced labile plasma iron**	Fluorescence-based assay indicative of total NTBI. See above				

*defined as levels that surpass cell innate capacity to produce sufficient ferritin or antioxidant that counteract formation of ROS
** See Methods on Reference n° 17: Wermke M et al, Lancet Haematol 2018.

synthesis and duplication.

The exceed iron, is deposited in the stable ferritin form. Excess LCI can enter the mitochondria and take part to increase the cellular ROS production through the Fenton's reaction. Usually the cell holds physiological adaptation mechanisms against the ROS level increases, mainly mediated by the activation of nuclear factor erythroid 2-related factor (Nrf2). Oxidative stress, normally produced in the cell, activates the Nrf2 pathway that is able to enter the nucleus and up-regulate the expression of antioxidant enzymes. Although Nrf2 can protect normal cells from oxidative stress, when the LCI level increases this mechanism becomes inadequate to control the ROS surplus and an oxidative stress state is established in the cell. Maintaining ROS at an appropriate level plays an important role in biological phenomena; increasing ROS or damage of the antioxidant system could lead to oxidative stress reactions and finally to cell damage (**Figure 1).**[9]

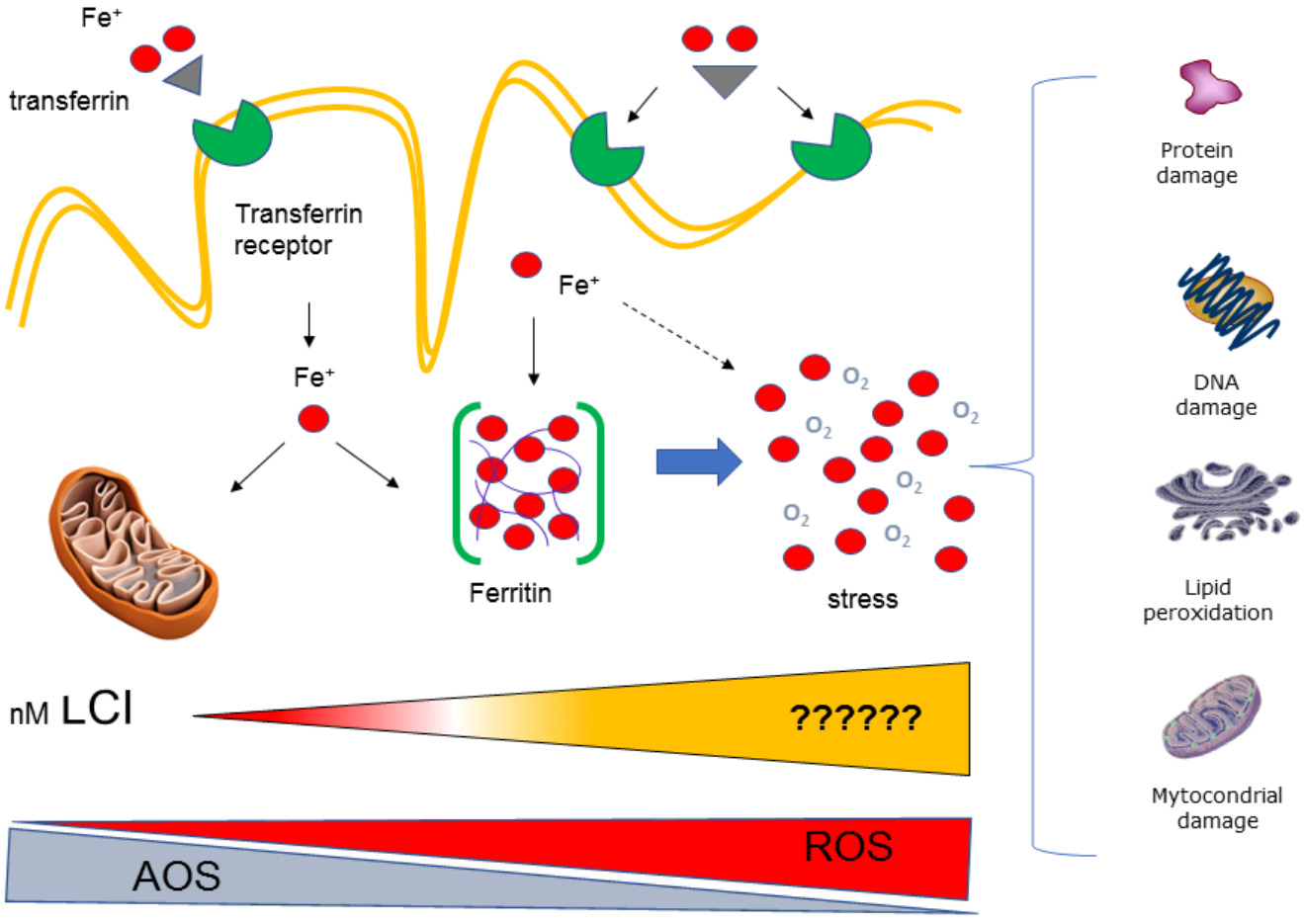
Nowadays, ferritin is considered a steady and not biological active form of iron, while LPI is considered the main trigger of cell damage more representative of the dynamic tissue damage. The scientific community is moving the iron disease from a "Bulky" disease, such as classically in thalassemia (based on quantitative iron parameters as ferritin, red blood cell transfusion number, MRI) to a "toxic" disease (based on active and dynamic biological markers as NTBI/LPI).

The mechanisms leading to iron dependent tissue / cells toxicity have been recently summarized by the following equation:

Iron toxicity tissue = Σ Tissue reactive iron x Genetics x Environmental Factors x Time

This formula proposes that tissue toxicity arises from both the quantity of toxic iron species (i.e., tissue reactive species = NTBI/LPI). The detrimental effects are further modulated by the host genetic characteristics, by the individual anti-oxidant mechanisms and by environmental factors.[10]

Free Iron Toxicity before Transplant. As far as the clinical counterpart of these biologic phenomena in transplantation, a clear example is the following: Pesaro's group divided TDT patients before HCT in three classes of risk predicting outcome based on: Liver fibrosis staging, Hepatomegaly presence, adequate iron chelation. This scoring system received criticism for its non-quantitative method mainly regarding hepatomegaly and the definition of adequate chelation.[11,12]



LCI: Labile cellular iron, AOS: antioxidant system, ROS: Reactive oxygen species

Figure 1.

Recently Angelucci et al revisited the Pesaro TDT score system based on the above-mentioned new concept of iron toxicity. It is evident that all the three risk factors were not quantitative direct markers of iron overload "per se", but indirect measures of intensity and extent of tissue exposure to toxic iron. Authors concluded that "adequacy of chelation" clearly means consistent and sufficient suppression of tissue reactive iron species (NTBI/LPI) over time. "Liver fibrosis" is definitely a marker of toxic iron exposure and environmental factors (i.e., viral infections) in the liver and "Hepatomegaly" reflects the extent of iron deposition and the time throughout exposure to NTBI and toxic reactive iron.[12]

At this time in all the published studies outside TDT, only the correlation between direct or indirect estimates of iron overload (mainly serum ferritin, transfusion burden and MRI values) and outcome parameters has been explored, while the duration of exposure to toxic iron species has not been taken into account.[4,5,13-16]

The first study that explored the LPI role in relationship with outcome was published by Wermke and colleagues in malignancies. They investigated the predictive value of both stored (MRI-derived liver iron content) and non-transferrin-bound-iron, defined as enhanced labile plasma iron (eLPI) (see **table 1**) on post-transplantation outcomes in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) . Their prospective, observational ALLIVE study recruited 112 patients transplanted in three years and showed that patients who had raised eLPI concentration at baseline, also had significantly increased incidence of non-relapse mortality at day 100 (33%) compared with those who had normal eLPI at baseline (7%) (P= 0.00034).They concluded that peri-transplantation eLPI-scavenging strategies could be explored in prospective international clinical trials for patients with systemic iron overload.[17]

Free Iron Toxicity During the Engraftment Period. It is supposed that LPI may be involved either in the occurrence of toxicity and other complications commonly observed in the early post HCT period independently from the underline disease and iron status.[5,18]

Firstly in patients who underwent HCT for several hematologic malignancies, it has been demonstrated how LPI levels, although normal at baseline measurements, increased substantially 48h after the start of conditioning with a peak around day 0, and remained increased until engraftment when it returned to baseline levels.[19]

The fast and substantial increase in LPI levels on Day 0 reflects a disruption of iron homeostasis by conditioning due to massive iron release by myeloablation and the temporary lack of iron uptake by the ablated erythroid system. In addition, it can be speculated that the conditioning-induced ablation of erythropoiesis could reduce the synthesis of erythroferrone, an erythroid hormone that suppresses hepcidin, favoring increased hepcidin levels that block the cellular iron efflux.[20]

Subsequently, reutilization of iron by restored erythropoiesis by engraftment leads to a substantial drop in LPI levels, but not in hepcidin levels, probably modulated also by inflammation. The interesting information is that ferritin levels that were already increased at baseline did not change throughout the engraftment period; so that cytotoxic chemotherapy and subsequent engraftment in HCT patients leads to changes in LPI but not in ferritin. For this reason LPI represents the marker that better reflects the modifications in iron status and could serve as a target of a possible chelation therapy in the early period of HCT.[21]

Similar results are shown by Duca et al regarding NTBI levels in thalassemic and leukemic patients.[22] NTBI levels were constantly higher at baseline in the thalassemia group but the relative increase compared to baseline was higher in leukemia patients (between 2.2- and 3-fold) than in thalassemia patients (between 1.6- and 2.5-fold). Early after transplant, concomitant with erythropoietic recovery, NTBI returned to respective baseline values. The marked increase of NTBI in serum after myeloablative chemotherapy was originally attributed to suppression of erythropoietic activity. Other possible sources of extracellular iron after myeloablative conditioning include lysis of erythroid bone marrow cells by cytotoxic injury. Also, this study confirms reduction of the iron uptake by erythroid precursors during and immediately after chemotherapy. Furthermore, in this case, NTBI decrement in all the patients after HCT could be explained by high iron requirement during allogeneic erythroid marrow rebuilding. High levels of iron overload, in the absence of chelation, are responsible for the persistence of NTBI high levels even after marrow reconstitution in thalassemia patients.[22]

A growing body of evidence describes how iron induced oxidative stress and increased ROS levels can modulate several signaling pathways (such as Protein kinase B, Tumor protein p53 and Wnt protein family,) which promote cell survival, avoid apoptosis, allow escape from growth arrest, and facilitate cancer transformation. Actually, ROS are involved in a complicated web of signaling networks where their generation is regulated by multiple pathways. Conversely, ROS act as signaling molecules for other signaling pathways such as PTEN, PTP1B, MAPK and NF-κB involved in different ways with the HSCs fate.[23,24]

In 2013, Chai and colleagues established an iron overloaded mouse model to investigate the effects of

iron overload on hematopoietic stem and progenitor cells (HSPCs). Results show that iron overload markedly decreased the ratio of immature hematopoietic cells and reduced HSPCs clonogenic capacity. Iron overload increased ROS levels of HSPCs through the NOX4/ROS/P38 MAPK signaling pathway.[25]

Similar results were found using bone marrow mesenchymal cells (BM-MSCs) in a similar murine model suggesting that iron can impair not only the HSPCs clonogenic capacity but similarly the quantity and quality of BM-MSCs and the bone marrow microenvironment as well.[26,27]

The effect of oxidative stress on hemopoiesis has been investigated in a murine transplant setting. A murine model was used to investigate the possible relationship between iron overload and engraftment post-allogeneic hematopoietic cell transplant. Donor bone marrow mononuclear cells (BM-MNCs) from iron overloaded mice and normal mice were transplanted into recipient mice. Flow cytometry analysis of peripheral blood cells from the recipient mice demonstrated that recipient mice of iron-overloaded donor had, after transplant, lower levels of myeloid B and T-lymphocytic lineage engraftments compared to the recipient mice of normal donor.[25]

A different conclusion was described by Okabe and colleagues,[26] who showed in an iron overloaded mouse recipient that oxidative stress could affect the engraftment of hematopoietic cells from a normal donor by modifying microenvironment and remarkably reducing expression of CXCL12, VCAM-1, Kit-ligand, erythropoietin and thrombopoietin. They concluded that iron overload can damage bone marrow stromal and other key organs (liver, kidney) and therefore, indirectly, hematopoiesis.

Interestingly, in almost all the above murine models, hematopoietic insufficiency improved by treating recipient mice with iron chelator or with the powerful antioxidant N-acetyl-cysteine (NAC), conveying that iron overload may be closely related to high oxidative stress.[25]

It is important to note that these experimental observations have not yet led to an operative approach. Little literature is available addressing the issue of peri-transplant chelation in thalassemia. These studies were designed with different objectives from the principles described above, but demonstrated the safety (no significant severe side effects) of iron chelation during the peri-transplant phase.[28] Fritsch et al utilized Deferasirox during the administration of conditioning regimens and it was found to be safe and reduced the appearance of LPI shortly after allo-HSCT in this preliminary study.[29]

The basic idea today would be to suppress, by adequate (not necessarily intensive) peri-transplant chelation, the NTBI/LPI increment occurring during transplant. Obviously, this rationale should be applied, at the moment, only in the context of a well-designed controlled clinical trial.

Free Iron Toxicity During the Early Transplant Phase (Before a Sustained Engraftment is Achieved). Tissues that have been damaged from iron toxicity before and during HCT gradually restore their functionality. Nevertheless, it is believed that, in some instances, ROS exposition persists also after transplant. In vitro tests on cardiac and endocrine cells have shown how after a prolonged and constant LPI exposition iron accumulates in organelles and increases ROS formation, affecting major cell functions such as permselectivity, electron transport activity, and viability. Again, iron chelation with Deferasirox was effective in reducing iron-induced cell damage and increasing viability of these cells.[30]

This model could also be applied after a sustained engraftment, not requiring blood transfusion, has been achieved. In fact, hematopoietic cell transplantation does not eliminate the iron excess acquired during previous years of thalassemia. Serum ferritin and the transferrin saturation slowly return to normal levels and only in patients with a very low iron burden before transplantation.[31]

Accordingly, with these observations, excess iron removing is essential after HCT. This recommendation is based on the evidence that progression of liver disease to cirrhosis has been documented in some patients in the years after transplantation. Data demonstrated a synergistic deleterious effect of Hepatitis C Virus (HCV) infection with iron overload with a multiplicative fibrogenic effect.[5,32] Recently a basic research paper has been published demonstrating a novel and intricate mechanism by which HCV interferes with the crosstalk between the Nrf2/ ARE-signaling, elevated ROS levels and autophagy. Basically HCV impairing Nrf2/ARE-signaling through the ROS increase create an amplified and deleterious hepatotoxic effect, favoring the HCV autophagy and its release and spread, worsening the preexistent hepatic failure.[33] Others synergisms with others damage factors (for example alcohol abuse) are likely.

Because a condition of iron overload leads to the release of reactive oxygen species, even in absence of ongoing transfusion need, defining iron overload post-transplant is essential and the gold standard for iron detection remain the liver iron concentration (LIC) because serum ferritin is particularly unreliable in this setting.

Recently the importance of LIC measurement by magnetic resonance imaging (MRI) has grown since it is non-invasive, rapid, and widely available. Today MRI techniques T2* and R2 are reported to have sensitivity and specificity of 89% and 80% in determination of LIC, respectively.[34]

Few studies examine the alterations of hepatic and myocardial T2* MRI values in TDT patients after HCT just before starting chelation therapy. The main study included fifty-two TDT patients with mean age of 7.6 years. Hepatic and myocardial T2* values before and 6 months after HCT were measured and analyzed. Results showed that there was not a statistically significant increase in myocardial T2* values after HCT, instead Hepatic T2* values significantly decreased after HCT showing an increase of the liver iron.[35]

Serum ferritin levels appeared to have a poor correlation with LIC in thalassemic patients after HCT . Ferritin can be a good screening test but a poor predictor of tissue iron overload.[36]

The above reported ALLIVE study in malignancies has been shown that only eLPI correlated with LIC (not ferritin neither hepcidin values) and patients with elevated eLPI baseline values had a worsening outcome due to an increased non -transplant related mortality.[17]

The hypothesis is that LPI/ NTBI might be used to assess the iron exposition status of organs after transplant, deciding initiation and duration of iron chelation and monitoring efficacy with the goals to protect organs from ROS exposition suppressing LPI and NTBI.

It is important to note that, in the current state of knowledge, it must be assumed that a normal transferrin saturation (i.e. 20-30%) excludes the presence of toxic iron forms of in the circulation and consequent progressing tissue damage; for this reason, it can be used as surrogate of normal ROS level.

Iron Chelation Therapy after Transplant in TDT. Because of the presence of effective erythropoiesis acquired by transplantation, phlebotomy is the preferred mechanism to remove excess iron after HCT. Phlebotomy is safe, inexpensive, and highly efficient. It can be started once engraftment is sustained and preferably after immunosuppressive therapy ending. Clinical improvement in liver and cardiac function has been demonstrated after iron depletion by phlebotomy in several instances.[37,38]

In transplanted patients undergoing phlebotomy, iron can be completely removed (final target: normal transferrin saturation, serum ferritin concentration <100 μg/L), and after this achievement, patients are free from iron overload and no maintenance therapy is required. Duration of treatment is strictly related to the magnitude of the iron overload, and it ranges from a few months to several years.

Two oral iron chelators have been tested in ß-TM (Deferiprone[39] and Deferasirox,[40] but only Deferasirox has been tested after transplantation. Reported cases of deferiprone-induced neutropenia in medically treated thalassemia patients raise concern for its use in the post-transplant setting.

The oral iron chelator Deferasirox has recently been tested in this setting on patients in prospective trials. Firstly, data from a prospective phase IV trials conducted by Vallejo et al showed that Deferasirox is efficient and safe in the post-transplant period in hematologic malignancies. Patients at least six months post-transplanted were treated with deferasirox dispersible tablets (DT) at a starting dose of 10 mg/kg/day for 52 weeks or until serum ferritin was less than 400 ng/mL on two consecutive occasions. A significant reduction from baseline in median serum ferritin and in liver iron concentration at 52 weeks was observed in the overall population. There were no drug-related serious adverse events.[41]

Similar results are recently described in the Thalassemic population. A prospective, phase II, multicenter, single-arm study evaluates the efficacy and safety of deferasirox-DT in patients age >2 to <18 years with TDT who had undergone HCT and had evidence of iron overload (serum ferritin >1000 μg/L; cardiac MRI T2* <20 ms, or liver iron concentration ≥5 mg/g). Patients received deferasirox at an initial dose of 10 mg/kg/day, with up-titration to a maximum of 20 mg/kg/day. There was a continuous decrease in median serum ferritin level from 1718.0 μg/L at baseline to 845.3 μg/L following 52 weeks of therapy ($P < .001$). There was also a significant decrease in median LIC and an increase in median cardiac T2* from baseline to week 52. A manageable safety profile was observed.[42]

A prospective randomized 1 year phase II trial comparing efficacy and safety of Deferasirox-DT versus the standard of care phlebotomy has been recently published in the population of transplanted thalassemia patients.[43] Deferasirox-DT starting dose was 10 mg/Kg/day increased till 20 mg/Kg/day during the trials. They showed that Deferasirox is efficient and safe post-transplant; no differences were reported in reducing serum ferritin ability in both arms.

The advantage of Deferasirox is that it is administrated orally. The disadvantages regard the possible renal and hepatic toxicity considering that such patients are receiving in this period cyclosporine and other drugs as well as its noticeable cost. In 2016 Jaekel et al showed that Deferasirox is efficient and safe, even in patients receiving cyclosporine. Deferasirox was initiated at a median of 168 days after HCT, with 84% of patients still on immunosuppression. The incidence of AEs appeared to be dose related, with 7.5 mg/kg/day of deferasirox-DT being the best-tolerated dose. They concluded that low-dose deferasirox is an effective chelation therapy after allogeneic HCT, with a manageable safety profile, even in patients receiving cyclosporine.[44] The recently

released film coated tablets (FCT) formulation promises to further increase tolerability.[45]

Reasons to select phlebotomies or deferasirox have been published.[13]

It is important to note that all transplanted patients after successful HCT face a normal life expectancy and therefore their target iron level must be a "normal "iron burden with normal ferritin and, mostly, normal transferrin saturation.

Conclusions. Reinterpreting transplant predictive factors in the light of the current advances in understanding iron homeostasis further supports the concept that the key to successful transplantation in thalassemia is regular and life-long chelation therapy to consistently suppress tissue reactive iron species and prevent tissue damage in the years before HCT. In the next near future, the suppression of the free iron forms (LPI, NTBI and ROS) could improve organ damage that could be important for the HCT and possibly even the gene therapy outcome.

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