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Invited review

Iron-mediated tissue damage in acquired ineffective erythropoiesis disease: It's more a matter of burden or more of exposure to toxic iron form?

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ABSTRACT

Iron is essential in cellular life, however, when in excess, it favors the production of reactive oxygen species (ROS) that, when overwhelm the physiological cellular antioxidant system, produce an oxidative stress state leading to cellular damages and organ failure.

What is not yet completely clear is whether the damage is related more to the amount of iron or to the duration of exposure to ROS. Various cellular pathways are sensitive to the detrimental action of ROS in a non-dose-dependent manner. In addition, different organs have a different capacity to respond to iron-mediated toxicity, suggesting that the toxicity thresholds are disease-specific and patient-dependent. The aim of this article is to review the recent understanding of the concept of exposure to free iron-mediated damage, comprehending the need to design protocols in which reducing organ exposure to ROS is the primary objective in order to prevent or delay the development of organ damage.

1. Introduction

Iron is an essential element for a multitude of biological processes. It is a transition metal with the ability to readily accept and donate electrons. This property allows iron to function as an oxidant or reductant in a large number of biochemical reactions. In fact, it is physiologically incorporated into the heme and into the iron sulphur complexes of many proteins and enzymes involved in transport, handling and utilization of oxygen such as haemoglobin and myoglobin, and of various non-heme proteins involved in mitochondrial activity, DNA synthesis and tissue repair. The same property that permits iron to gain and lose electrons can result detrimental if involve oxygen reactions. This occurs when iron is in excess and is present in blood and cells as unbound "free" iron; this causes the generation of reactive oxygen species (ROS) that can lead to cell and tissue damage. Therefore, iron uptake, utilization, storage and export must be tightly regulated to maintain iron homeostasis and to avoid the development of iron deficiency, anemia and tissue hypoxia by one hand, and iron- overload related toxicity through generation of ROS, by the other. This challenging task is mainly performed by the iron-regulatory protein (IRP)/iron responsive element (IRE) system in the cell, and hepcidin-ferroportin axis at systemic level.

In physiological conditions, iron entry through the cell membrane is mediated by the transferrin receptor. Once inside the cell, iron can be stored by binding with ferritin or carried to the mitochondria to be used in heme synthesis and in the formation of proteins containing iron-sulphur clusters, the last option, if it is not used, is to be exported from the cell [1].

The iron that inside the cell takes part in cellular life is known as labile cellular iron (LCI); the LCI level is closely regulated by a sensitive and efficient iron homeostasis control system that makes it possible to

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maintain adequate levels of free iron in the plasma and cells. When it is present in excess, and therefore cause of increase in intracellular oxidative stress, the intracellular antioxidant system rebalances the levels of oxidative stress, to guarantee normal cell function and avoid iron-mediated cellular damage [2]. What is still partially clarified is if the iron mediated damage is caused by a "quantitative" level of iron loaded or if the damage could be start previously just because of exposition to free toxic form of iron.

2. Iron homeostasis from physiology to pathology

2.1. Cellular and systemic regulation mechanisms

Although cellular iron homeostasis is under a multiple step control, the post-transcriptional regulation mediated by the binding of IRP1 and IRP2 to cis-regulatory mRNA IREs has emerged as central. The IRE/IRP system regulates the expression of several mRNAs encoding proteins for iron acquisition [transferrin receptor 1 (TFR1), Divalent-Metal-Transporter (DMT1)], storage (H- and L-ferritin), utilization [hypoxia-inducible factor 2 (HIF2α)], and export (ferroportin), and additional mRNAs [3,4]. IRE/IRP complexes formed within the 5' untranslated region (UTR) of an mRNA (e.g., ferritin, ferroportin) inhibit translation, whereas IRP binding to IREs in the 3'UTR mRNA of TFR1 and DMT1 prevents degradation. The fine regulation of intracellular iron levels is mainly achieved by means of a divergent but coordinated regulation of iron-uptake, utilization, storage and export iron proteins. Under condition of iron deficiency, IRPs actively bind to the iron responsive elements, located in the 3' or 5' UTR mRNAs, and stabilizes TfR1 and DMT1 mRNAs increasing the uptake of iron, while it decreases translation of ferritin and ferroportin mRNAs, increasing the availability of iron. Conversely, high iron levels decrease iron responsive elements-binding affinity, favoring iron sequestration and export over uptake by increasing ferritin and ferroportin expression.

The body iron content is about 40–45 mg/kg in women and 50−55 mg/kg in men of which approximately 70 % is contained in erythroid bone marrow and red blood cells, bound to haemoglobin. The control of systemic iron levels occurs through the regulation of iron acquisition, recycling and storage, because there is no known regulated form of iron excretion (Fig. 1). The main actor of systemic iron homeostasis is hepcidin through the binding to its receptor and cellular iron exporter ferroportin, regulating intestinal iron absorption and macrophage iron release in order to maintain transferrin iron saturation adequate to body requirement. In physiological condition, daily intestinal non-heme iron absorption is limited by the hepcidin- ferroportin axis to 1−2 mg to balance the obligate losses through cellular exfoliation. However, it can be modulated in case of increased iron needs. In the blood, iron is transported to TFR1 at the cell surface followed by endocytosis of the Tf-Fe2/TFR1 complex, release in the cytosol through DMT1 for utilization or storage. As supported by the hypo-transferrin human and murine models, the Tf-Fe2/TFR1 binding seems not dispensable for the erythroid bone marrow, while other tissues can acquire iron even through less specific membrane transporters [5].

Ferroportin is a membrane protein expressed in all cells and is the only known iron exporter in mammals. Ferroportin needs copper-ferroxidases to release iron to plasma transferrin, hephestin in enterocytes and ceruloplasmin in macrophages and hepatocytes. Although ferroportin gene transcription and translation is modulated by a number of multi-layered signals, the activity of ferroportin on cell membranes is predominantly governed post-translationally by hepcidin [6].

Hepcidin, a small peptide that is mainly produced by hepatocytes, regulates intestinal iron absorption and iron release from storage cells by binding and blocking ferroportin either via degradation or via occlusion, thus exerting a general inhibitory effect on iron release within the body [7]. In physiological conditions, hepcidin production is tightly regulated in response to different signals, e.g. bone marrow iron requirements, hypoxia, transferrin saturation, iron stores, and inflammation through

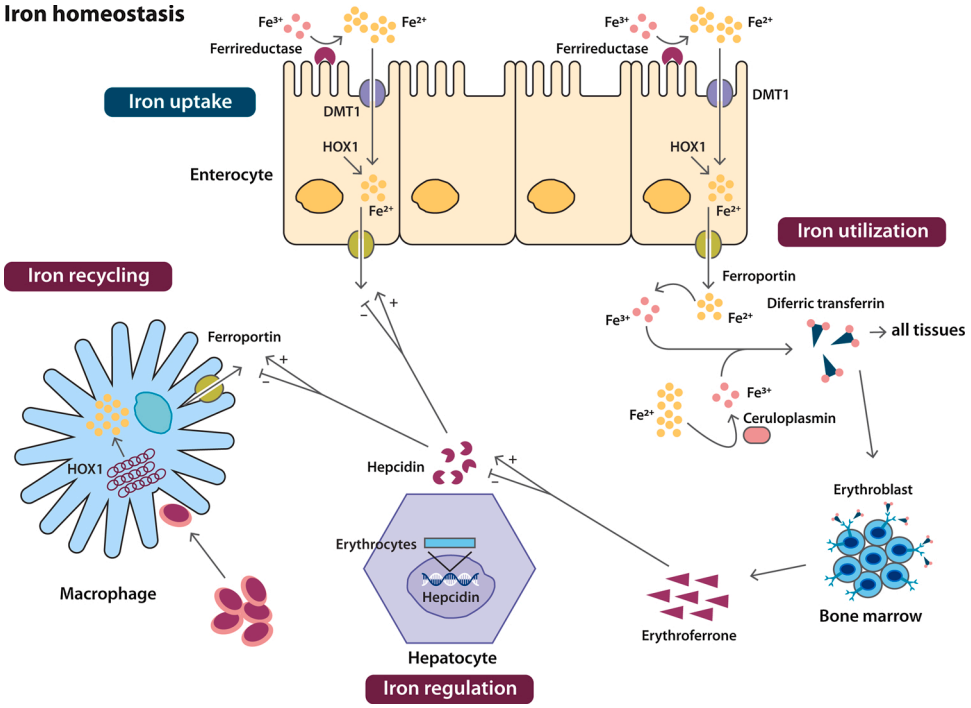

Fig. 1. Iron homeostasis.
Iron is transported by transferrin (TF) in the blood, and released through the binding of diferric-TF (TF-Fe2) to transferrin receptor (TFR)-1 at the erythroblasts surface followed by endocytosis of the Tf-Fe2/TFR1 complex to produce new haemoglobin during erythropoiesis in the bone marrow.
The hepcidin-ferroportin axis regulates the intestinal non-heme iron absorption to balance the limited daily loss of 1−2 mg of iron through cellular exfoliation (in particular of the intestinal epithelium).
Hepcidin, a small peptide that is mainly produced by hepatocytes, regulates intestinal iron absorption and iron release from storage cells by binding and blocking ferroportin either via degradation or via occlusion, thus exerting a general inhibitory effect on iron release within the body. Ferroportin is a membrane protein expressed in all cells and is the only known iron exporter in mammals. Ferroportin needs copper-ferroxidases to release iron to plasma transferrin, hephestin in enterocytes and ceruloplasmin in macrophages and hepatocytes. Although ferroportin gene transcription and translation is modulated by a number of multi-layered signals, the activity of ferroportin on cell membranes is predominantly governed post-translationally by hepcidin. Increased hepcidin expression limits iron absorption while its reduction allows greater iron absorption and macrophage iron release. Ferroportin-mediated iron release from duodenal cells and macrophages uptake, increase in plasma transferrin saturation, appearance of non-transferrin-bound iron (NTBI) and labile plasma iron (LPI), and tissue iron overload.

different signaling pathways [5,8]. Increased hepcidin expression limits iron absorption while its reduction allows greater iron absorption and macrophage iron release. Inherited defects of hepcidin regulator proteins [HFE, hemojuvelin (HJV), and transferrin receptor 2 (TFR2)] as occurs in hemochromatosis type 1, 2 and 3 and erythropoietic expansion due to ineffective erythropoiesis (e.g.: thalassemia, diserythropoietic anemias) suppress hepcidin synthesis.

Recently another actor of iron homeostasis has been elucidated; erythroferrone (ERFE), a TNFα-like protein released by mature erythroblasts in condition of enhanced erythropoiesis, is considered the major candidate of erythropoiesis-induced hepcidin suppression [9,10].

In physiological condition, during stress erythropoiesis like hemorrhages or hemolysis, anaemia causes increased erythropoietin (EPO) secretion by the kidneys. EPO stimulates erythroferrone secretion that inhibits hepcidin transcription, and provides adequate iron for erythropoiesis and other body requirements. Hepcidin suppression, in turn, cause higher intestinal iron absorption, iron recycling from splenic macrophages and iron release from hepatic stores. As a result, circulating iron increases, allowing increased iron availability for compensatory erythropoiesis.

This systemic control of iron is profoundly changed in anemias with congenital or acquired ineffective erythropoiesis, where erythroblasts do not successfully differentiate into mature erythrocytes so that iron is over mobilized but erythrocyte production cannot increase and the additional iron is not utilized, increasing the plasma transferrin saturation. When transferrin uptake capability exceeds, non-transferrin-bound iron (NTBI) and labile plasma iron (LPI) appear resulting in cellular iron toxicity [11].

2.2. Cellular iron toxicity

NTBI and, in particular, its biologically active sub-component LPI are able to enter the cell without using the TFR1 as a gateway, possibly involving the transporter SLC39A14 (ZIP14) in hepatocytes and acinar pancreatic cells, and ZIP8 and L-type calcium channels in cardiomyocytes, thus increasing the intracellular labile iron pool. Inside the cell, excess labile cellular iron comes into contact with intracellular oxygen and ROS are produced. Increased ROS production is a process that occurs physiologically in stress conditions; however, the efficient antioxidant system rebalances oxidative stress levels, thereby guaranteeing normal cellular function and avoiding iron-mediated cell damage. In abnormal situations, the increase in ROS causes a biologically toxic action on a molecular, cellular and tissue level.

The ROS generated by an excess of “free” iron cause tissue damage by means of various mechanisms: a) they can induce apoptosis (programmed cell death) through the activation of both the intrinsic and extrinsic pathways, thereby also supporting the activity of the pro-apoptotic BAD and BAX genes, to the detriment of the antiapoptotic BCL-2 gene; b) they induce autophagia, through mTOR inhibition; c) they directly induce necroptosis; and d) they induce ferroptosis, through increased lipid peroxidation. Although this last iron-mediated cell death mechanism has yet to be completely clarified in blood disorders, it has been extensively described in neurodegenerative conditions, liver diseases and solid tumours [12].

The ROS also cause telomere shortening, the activation of various oncogenes (RAS, AKT) and the inactivation of onco-suppressors, such as TP53, thereby causing heightened genomic instability. The ROS produced in excess in the presence of free iron induce increased angiogenesis through the production of VEGF, VEGF receptor up-regulation, and the regulation of various genes, such as those encoding for the metalloproteases, which are involved in this process [13].

The only cellular system known to counter the increase in ROS is the antioxidant system, which however, in certain tumours such as myelodysplastic syndromes is less efficient than in healthy controls [14].

Although iron toxicity is associated with “free” iron and the consequent production of ROS, very few laboratories are currently equipped to determine NTBI, LPI and ROS and even these not outside of clinical trial protocols. ROS production can be indirectly assessed by measuring markers of lipid, protein and DNA peroxidation, and mitochondrial damage. However, transferrin saturation is currently the easier and more reliable indirect marker of ROS productions; it has been extensively shown that NTBI and LPI are found in circulating blood when transferrin saturation exceeds 70–80 % [15,16]).

Iron is stored in humans as ferritin and hemosiderin. While ferritin is stable this is not the case for hemosiderin that results from degradation of ferritin as a mixture of Fe3+ cores and peptides. It follows that increased iron stores continuously produce free iron forms which, if excessive, are no more linked to serum transferrin.

As in humans there is no physiological mechanism for eliminating excess iron, when accumulation occurs, the accumulated iron must be removed by phlebotomy treatment or, in the presence of concomitant anaemia, the use of medicinal products able to chelate the iron and remove thereby reducing its levels in the body [17–19].

2.3. Main causes of iron overload in acquired ineffective erythropoiesis disease

Iron overload can be developed due to different causes, the main of them are summarised below and in Fig. 2.

2.3.1. Transfusion dependency

Excessive iron import via the parenteral route, generally because of repeated blood transfusion in patients with various forms of severe acquired or hereditary anaemias.

In medical conditions requiring frequent blood transfusions, iron initially accumulates in macrophages and subsequently, once the limited containment capacity of this compartment has been exhausted, it is released into the plasma bound to transferrin. When the binding capacity of transferrin has been saturated, NTBI and LPI are generated, with the consequences described above. These conditions include haemoglobin disorders, myelodysplastic syndromes, myelofibrosis and aplastic anaemia.

2.3.2. Ineffective erythropoiesis

Ineffective erythropoiesis is defined as the inability to produce an adequate number of mature erythroid cells, the result is a hyperplasia of immature erythroid cells at the bone marrow level but peripheral anemia. In anemias with ineffective erythropoiesis like myelodysplastic syndrome (MDS), most erythroblasts do not successfully differentiate into mature erythrocytes, leading to anemia and increased erythropoietin (EPO) production by the kidneys. Under the EPO signal, there is an erythroblastic hyperplasia response that causes an increase in the production of the EPO-STAT5 dependent hormone erythroferron. ERFE secretion is increased both because of EPO but also of the ERFE-secreting erythroblasts. ERFE is secreted into the circulation and, in the liver, it has the property of suppressing the production of hepcidin.

Over the course of time, the combination of tissue hypoxia, increased erythropoietin and ineffective erythropoiesis creates a vicious cycle that may ultimately lead to a massive expansion of erythroblasts [11]. As a result, circulating iron increases allowing increased iron availability for erythropoiesis but erythroblasts do not successfully differentiate into mature erythrocytes, leading to saturation of transferrin and free forms of iron appearing.

In other words, iron is mobilized but erythrocyte production cannot increase and the additional iron is not utilized, generating NTBI and LPI that have redox activities. They enter into the cell and react with intracellular oxygen with production of ROS.

This model explains the presence of iron overload in all acquired and congenital conditions characterised by ineffective erythropoiesis [11] also in absence of transfusion dependency.

As there is currently no univocal biological or biochemical marker of dyserythropoiesis, it is difficult to assess to what extent

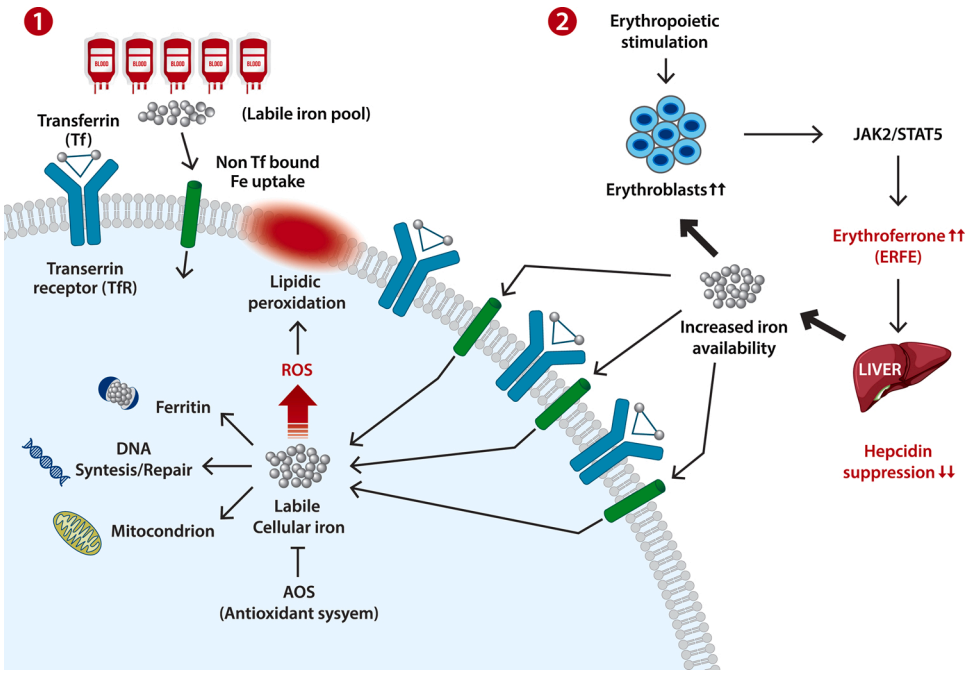

Fig. 2. Mechanisms of iron loaded in acquired ineffective erythropoiesis disease.
1) Transfusion dependent iron overload: in medical conditions requiring frequent blood transfusions, iron initially accumulates in macrophages and subsequently, once the containment capacity of this compartment has been exhausted, it is released into the plasma bound to transferrin. When the binding capacity of transferrin has been saturated, NTBI and LPI are generated.
2) Ineffective erythropoiesis: anaemia increases the production of erythropoietin, which in turn stimulates the production of erythroferrone both directly and indirectly (through erythroblast hyperplasia). The increase in erythroferrone in turn suppresses hepatic hepcidin production, which causes a physiological increase in duodenal iron re-uptake and the release of iron deposited in the spleen and liver. However, the more abundantly available iron is not used correctly in erythropoiesis, because it is defective, and it does not offset the state of anaemia, thereby creating a vicious cycle. Therefore, instead of being used, it is deposited in the tissues as NTBI and LPI.

dyserythropoiesis affects iron overload and the increase in the free toxic forms of iron. Moreover, a marker of dyserythropoiesis could indirectly indicate the oxidative state of the main excess iron storage organs (liver and heart). Furthermore, the correction of dyserythropoiesis using novel medicinal products (such as the TNF-beta inhibitors) could improve the use of the iron made available on a systemic level by the ameliorate effective erythropoiesis [20]

A recent study demonstrated that the accumulation of LPI associated with dyserythropoiesis in the forms of MDS with ring sideroblasts not transfusion-dependent, has a similar impact on survival to that observed in patients with other types of MDS receiving transfusions [21]. The reason is that MDS with ring sideroblasts and SF3B1 mutation is considered the MDS subtype more susceptible to iron toxicity secondary to the higher level of ineffective erythropoiesis in particular for production of alternative transcript encoding erythroferron and consequent constant suppression of hepcidin which in these forms of MDS causes increased iron accumulation [22,23].

3. Tissue-specific exposure to the toxic forms of iron

Although excess iron damage involves all the body's tissues, only some become the main targets of iron-mediated damage. What is not yet completely clear is whether the damage that occurs in these tissues is related more to the amount of free iron able to produce ROS, and therefore organ damage, or to the duration of exposure of the various tissues to ROS. Undoubtedly this is the reason why is difficult to promptly prevent the iron damage.

The main target of iron toxicity is the liver, due to the predominant role it plays in the metabolism of this metal. Although iron accumulation in the heart can only be observed at a later stage (after > 70 units of packed red blood cells) [24], heart failure is a serious and potentially fatal complication associated with iron-mediated toxicity and damage. In this organ, the excessive production of ROS caused by intracellular iron causes abnormalities in the mitochondrial respiratory chain that, in turn, translates into muscle fibre degeneration, hypertrophy, and the onset of arrhythmias and decompensated heart failure [25,26].

In addition, previous studies in animal models and humans have also revealed a relationship between iron accumulation and atherosclerosis [27,28].

The endocrine system, especially the pituitary, thyroid and parathyroid glands, and the pancreas, can be damaged by the accumulation of iron, with the consequent development of hypogonadism, infertility and diabetes mellitus [29,30].

Last, the bone marrow can also constitute a target for iron toxicity, with negative effects on erythropoiesis and myelopoiesis [31–34].

Indeed, we know that the presence of excessive amounts of free iron represents an element of toxicity for tissues and organs [35], however, it is important to highlight the fact that the free forms of iron (LPI, NTBI) are highly reactive and are able to enter the cells in an uncontrolled way, thereby altering the intracellular balance between use and conservation of the labile iron pool and the formation of ROS, with consequent organelle damage and cell death. Consequently, iron toxicity can develop well before there is clear evidence of iron deposition in the individual organs. The non-conventional tissue entry channels for free iron are to some extent described in Table 1.

The main hypothesis is that cytotoxicity is not merely and directly caused by stored iron, but by an abnormality of the dynamic balance between the iron stored in the body and the iron used in biological processes [36]. It would appear sufficient to cause an alteration in the balance at cellular level that triggers the induction of oxidative stress, which leads to cytotoxicity.

Therefore, in order to promptly determine a condition of iron-mediated toxicity, even before there are instrumental findings of iron

Table 1
Tissues non-conventional channels for free iron.

Protein	Gene	Organ/tissue
ZIP14 (ZRT/IRT-like protein 14)	SLC39A14	Liver (Hepatocytes) Pancreas (Acinar cells/ β-cells)
LTCC (L-type calcium channel)	Cav 1.2/ 1.3	Heart (Cardiomyocytes)
TTCC (T-type calcium channel)	Cav 3.1	Heart (Cardiomyocytes)
DMT1 (divalent metal-ion transporter 1)	SLC1 1A2	CNS (Astrocytes, Microglia)
ZIP 8 (ZRT/IRT-like protein 8)	SLC39A8	CNS (Neurons)
TRPC6 (transient receptor potential cation channel subfamily C member 6)	TRPC6	CNS (Astrocytes)

deposition, the possible presence of excess free iron in the plasma must be considered. NTBI is rarely present and detectable in the body when the level of transferrin saturation is <70 %, but it progressively increases when values exceed 70–75 % [21,37,38]. In patients with MDS, high NTBI and LPI values (>the lower limit of detection) have been associated with transferrin saturation values of >70 % and >80 %, respectively [21]. Furthermore, the classic biochemical and diagnostic markers of iron overload do not always correlate with cellular/tissue toxicity. For example, high levels of NTBI not necessarily associated with iron overload have been observed in patients receiving stem cell transplants, especially during and immediately after the conditioning phase [39].

The pathogenesis thresholds of free or deposited iron-mediated damage may vary depending not only on the tissue involved, but also on the efficacy of the antioxidant system, comorbidities and age.

Coates et al. [35] developed an equation for the assessment of tissue iron toxicity and described how iron-mediated damage derives from the sum of the effect of ROS multiplied by genetic susceptibility, microenvironment and duration of exposure.

In other words, according to this Author, tissue damage does not depend on just one of the aforesaid factors, which explains why it differs in the various iron overload condition, which is not only organ-dependent, but also patient-dependent. Furthermore, the time factor, intended as the duration of exposure to the toxic substance, becomes fundamental.

The various factors are analysed below:

3.1. Quantity of toxic iron in the tissue

The damage caused by the accumulation of iron depends above all on the amount of free iron (NTBI and LPI) levels in the various tissues [40]. However, this effect would appear to have a different impact in the different tissues, due to the differences in tissue-specific susceptibility to iron-mediated damage (transferrin receptors number, antioxidant capacity, type of ferritin present). For example, hepatic ferritin (containing more L-ferritin) is better suited to iron storage than cardiac ferritin (containing more H-ferritin). This variable tissue susceptibility translates into different correlates between the amount of iron overload and the severity of organ damage in different tissue as shown in the heart where toxic iron is ten times lower than in the liver [41,42]. The high incidence of pituitary hypogonadism in juvenile haemochromatosis and transfusion-dependent thalassaemia [43,44], suggests that the pituitary gland is more exposed to iron toxicity damage during certain stages of development, such as puberty, during which, for example, the level of transferrin receptor expression in the gonadotropic cells is higher and the capacity to accumulate iron is greater. On the other hand, it is also reasonable to postulate that the reduction in antioxidant capacity associated with age and certain types of cancer may expose the tissues to a risk of damage at lower iron levels.

3.2. Genetic factors

Glutathione polymorphisms could also play a role in the development of iron-mediated tissue damage. By inhibiting the membrane system that converts cystine into cysteine and generating glutathione (GSH), the inducers of ferroptosis (such as erastin) cause a decrease in reduced glutathione, with a consequent increase in cell death. Genetically more efficient systems of glutathione-related detoxification could therefore be more advantageous in some subjects than in others. It is also likely that polymorphisms that favour fibrogenesis may have a significant impact on the extent of organ damage. Protective factors (metalloproteinases) associated with genetic factors and age may also play an important role in the onset of tissue damage [45].

3.3. Environmental factors

This kind of factor may include: a) dietary deficiencies, present in chronic alcoholics and those with severe vitamins or oligoelements deficiencies (for example thiamine, selenium, vitamin D and vitamin C deficiency), which have a negative impact on iron-mediated tissue damage by reducing the correct elimination of the ROS; b) the use of medicinal products that are harmful for the target tissues (for example chemotherapy drugs, such as anthracyclines, which cause myocardial damage); and c) comorbidities. Age is another factor that influences organ damage because it is associated with comorbidities and because ageing is associated with a decrease in protective and repair factors.

3.4. Duration of exposure to toxic iron

Tissue damage is also associated with the duration of exposure to free iron and the consequent increased production of ROS, as has been extensively demonstrated in different human models of iron overload in which organ damage requires years of exposure to toxic iron before presentation. Hepatic cirrhosis, for example, requires high liver concentrations of iron (LIC) and lengthy exposure to excess iron to develop [18,46]. However, in the more severe and earlier forms of iron accumulation, such as juvenile haemochromatosis and transfusion-dependent thalassaemia, cardiac and pituitary damage may be present even in the absence of hepatic cirrhosis [43,44]. This demonstrates that the time for developing iron-mediated damage may vary from one tissue to another. The authors postulate that lengthy exposure to slightly increased levels of ROS may have the same effect as short exposure to high levels of ROS.

3.4.1. Hepatic exposure to the free radicals of iron

Liver is the first organs involved in the iron loaded because of its pathways of iron uptake and efflux compared to the other organs. In the liver, the excess iron induces fibrosis that may progress to cirrhosis and, consequently, to hepatocellular carcinoma [47].

Transferrin bound-iron (TBI) is taken up by both TFR1 and TFR2.

TFR1 is expressed in most tissues but at much higher levels in erythroid precursors and liver, TFR2 is exclusively expressed in the liver and at levels higher than those of TFR1 but the affinity of TFR1 for iron is 25 times higher than that of TFR2 [48].

When NTBI and LPI appear in the plasma, hepatic uptake of this labile iron form through non-transferrin-dependent pathway is rapid and efficient. In mice model this uptake is thought to involve DMT1 and zinc transporter ZIP14 on hepatocyte cell surface.

Cells normally control the uptake of iron by modulating the expression of TFR1 accordingly to needs, but in state of iron excess NTBI uptake is uncontrolled. The ability of the liver to load both TBI and NTBI may explain the very rapid loading of iron in the liver in human.

3.4.2. Cardiac exposure to the free radicals of iron

Preclinical models recently showed that LPI and NTBI can enter the mitochondria of the myocardial fibres through the T-type and L-type calcium channels in an uncontrolled way, thereby affecting fibre contractility and, consequently, myocyte kinetics. Iron induced mitochondrial abnormalities regress almost completely after the administration of a mitochondrial calcium channel blocker (RU360) or iron chelator [25].

These preclinical models demonstrate how myocytic fibre kinetics and impulse propagation are affected far earlier than the presentation of systolic dysfunction that can be detected by echocardiography [28].

In the transfusion-dependent thalassaemia setting, cardiac MRI T2* values allow patients to be classified according to the severity of iron overload (MRI T2* relaxation time >20ms: normal; 10–20 ms: mild/moderate; <10 ms: severe), and and to define the corresponding risk of developing heart disease such as such as decompensated heart failure or arrhythmia (Poggiali et al., 2012) [49]. The MRI T2* relaxation time

threshold values associated with toxicity and iron overload were identified in patients with thalassaemia and have been validated in this patient setting alone. To date no specific cut-offs have been defined for the other chronic transfusion-dependent forms of anaemia, in particular for elderly patients with other cardiac comorbidities.

Thus, the threshold values defined in thalassemia have been translated tout-court in other situations of iron overload such as, for example, myelodysplastic syndromes. Although cardiac iron overload at MRI generally occurs after 75–80 units of RBS in MDS patients, they often manifest cardiac and/or cardiovascular complications even before the evidence of iron overload at organ level (ferritin elevation occurs after just approximately 20 units) [24,50]. This is because cardiac dysfunction, like other kinds of iron-mediated organ damage, may not depend solely on the concentrations of iron in the tissues, but also on the presence and duration of exposure to free iron (NTBI and LPI), co-factors such as anaemia and other, age-related comorbidities and depletion of antioxidant protective molecules.

All these factors might contribute to the development of clinical complication even at concentration below the MRI detectability threshold.

3.4.3. Bone marrow exposure to the free radicals of iron

Even at haematopoietic cell level, the toxic effect of iron on haematopoiesis would appear to be associated more with the presence of LPI than with the total quantity of iron deposited, as is indirectly demonstrated by the beneficial action of iron chelation on the iron-mediated oxidative stress that precedes the decrease in serum ferritin [51].

Stem cells reside in the haematopoietic niche and usually, during their quiescent phase, they are found in the osteoblastic region, which has a low concentration of oxygen. Following a partial pressure of oxygen gradient, they leave the osteoblastic region and the quiescent phase to proliferate and mature in the vascular region of the haematopoietic niche. The oxygen gradient correlates in preclinical studies with the ROS gradient. ROS levels are low in quiescent cells (in conditions of hypoxia) and subsequently gradually increase during proliferation and maturation [36]. Mouse model data show that the increase of iron concentration induces the production of ROS that may affect cell maturation, proliferation and clonal capacity and lead to cell death [31]. The same principle has been also demonstrated for the bone marrow microenvironment (mesenchymal/endothelial cell proliferation abnormalities) and it is described how decreases the expression of the cytokines and growth factors involved in these process [32]. In murine transplant model, iron overload is associated with engraftment difficulties because of the iron-induced oxidative stress at both stem cell and microenvironment level [52].

It has also been shown that ROS act as “second messengers” for molecules known as “redox sensors” that are involved in pathways that intervene in the destiny of the stem cell. Thus, an abnormality in these pathways, associated with an increase in ROS levels or in the duration of exposure to them, may affect the correct function of the stem cell [34].

4. Which is the ideal iron chelation approach?

The aim of iron chelation treatment is to reduce the level of iron in the body both by removing excess iron deposits (organ overload) and by eliminating the forms of free iron (LPI/NTBI) that, as we have seen, are extremely dangerous when present in excess.

In a meta-analysis, iron chelation was seen to be associated with longer survival in patients with MDS (low-risk MDS) [53], therefore suggesting that patients receiving iron chelation therapy may live longer.

A five-year non-randomized prospective analysis performed on the data of 599 patients with low-risk MDS and transfusion-induced iron accumulation showed a higher mortality rate in patients that did not receive iron chelators (73.3 %) than in those treated with iron chelation therapy (62.2 %, $p = 0.0039$) and even more if treated for $\geq$6 months (59.6 %, $p = 0.001$) [54]. The survival of patients who received iron chelation therapy (86.3 and 98.7 months, respectively) was significantly longer than in those who did not receive iron chelators (47.8 months) [54]. This study also analysed and compared the causes of patient death showing that progression to acute myeloid leukaemia (AML), infections and tumours were the causes that most explained the higher mortality rate observed in non-iron chelated patients [54].

To further investigate the relationship between survival and iron chelation, the TELESTO study was designed and initiated in 2009 aiming to show whether iron chelation therapy with deferasirox has clinical benefits in transfusion-dependent low-risk MDS patients. This is the first and the only randomised, double-blind, prospective study done in this patient setting. TELESTO enrolled 225 patients with low/Int-1-risk MDS who were randomised to receive placebo or iron chelation therapy with deferasirox (random 1:2); the primary endpoint was event-free survival (EFS) (such as death and cardiac or hepatic events). Estimated EFS at 3 years was 61.5 % (95 % CI: 52.2–69.6) with deferasirox and 47.3 % (95 % CI: 31.8–61.3) with placebo [55]. The TELESTO study data confirm, for the first time in the myelodysplasia setting, the clinical benefit of removing iron toxicity with deferasirox, thereby achieving clinical survival outcomes such as EFS. It remains to be seen to what extent cardiac and hepatic death impact EFS in patients not receiving iron chelation therapy, as the TELESTO study was not “statistically powerful” for answering this question although a trend emerged in considering cardiac disease as the main pathological factor impacting EFS.

The aforesaid studies clarify what is expected for iron chelation therapy, i.e. best clinical outcomes and delayed cardiac and hepatic events, in particular.

The limit of the above studies is that, as all the retrospective and prospective studies, including the TELESTO study, were designed using the same inclusion criteria and drug doses as those established in the clinical trials evaluating the efficacy and safety of iron chelators in thalassaemic patients. Considering preclinical data showing that exposure to NTBI and LPI in elderly patients with tumours including MDS exerts greater damage than in young patients with thalassaemia, it remains to be established if in such a kind of population the iron-mediated damage started earlier than in thalassemic patients.

With the aim to investigate this issue, two prospective studies - one Italian and one French – have been designed with the primary objective of suppressing the NTBI and LPI forms of free iron before or at the onset of transfusion dependency (and so before the formation of organ deposits) with a minimum dose of deferasirox [56,57].

A recent study published by M. Hoeks and colleagues [58] confirms the clinical importance of reducing the duration of exposition to free iron form LPI and starting an early iron chelation. They showed that LPI levels above the low level designed (LLOD) are associated with a lower overall and progression-free survival irrespective to transfusional iron load in low risk MDS patients [58]. They highlighted that the indirect production of cellular ferroptosis levels MDA (malondialdehyde) were within the reference range in the non-ring sideroblastic MDS with transfusion independency group but above the upper limit of the reference range in all other subgroups with the highest levels in the ring-sideroblastic MDS with transfusion dependency group.

This means that ring sideroblastic MDS have the highest levels of markers that reflect iron toxicity also in absence of transfusion dependency because of the highest level of iron loaded came from dyserythropoiesis, as previously described.

Currently the LPI measurement is not standardized and suitable in the clinical practice, but transferrin saturation is an indirect value of free iron form in the plasma and so of tissue iron toxicity. The goal of a correct iron-chelation therapy should be a transferrin saturation $<$ 65–75 % to avoid NTBI and LPI production.

5. Conclusions

Iron is an essential metal in cell life, but when in excess causes the development of non-transferrin-bound iron and its biologically active labile component that favours the production of reactive oxygen species that, when present in excessive quantities, are cytotoxic.

Different organs have a different capacity to respond to iron-mediated toxicity, suggesting that toxicity thresholds are disease-specific, tissue-specific, and patient-dependent. So far, cut-offs have only been identified and classified in transfusion-dependent thalassaemia setting. In all the other acquired dyserythropoietic disease they are still to be defined, but currently the cut-offs used in thalassaemia are commonly adopted, which is probably both inadequate and misleading.

What is not yet completely clear is whether tissue damage is related more to the amount of free iron able to produce ROS or to the duration of exposure of the various tissues to ROS in a non-dose-dependent manner.

The understanding of the importance of the exposure to free iron in inducing iron-related damage rather than the consolidated concept of accumulation has urged the scientific community to design study protocols in which iron chelation has the primary objective of reducing organ exposure to LPI and NTBI at an early stage, in order to avoid or at least delay the development of organ damage.

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Authors' contribution

All the authors contributed equally to concept and write the manuscript.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

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