Non-transferrin bound iron: A key role in iron overload and iron toxicity

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Abstract

Background: Besides transferrin iron, which represents the normal form of circulating iron, non-transferrin bound iron (NTBI) has been identified in the plasma of patients with various pathological conditions in which transferrin saturation is significantly elevated.

Scope of the review: To show that: i) NTBI is present not only during chronic iron overload disorders (hemochromatosis, transfusional iron overload) but also in miscellaneous diseases which are not primarily iron overloaded conditions; ii) this iron species represents a potentially toxic iron form due to its high propensity to induce reactive oxygen species and is responsible for cellular damage not only at the plasma membrane level but also towards different intracellular organelles; iii) the NTBI concept may be expanded to include intracytosolic iron forms which are not linked to ferritin, the major storage protein which exerts, at the cellular level, the same type of protective effect towards the intracellular environment as transferrin in the plasma.

Major conclusions: Plasma NTBI and especially labile plasma iron determinations represent a new important biological tool since elimination of this toxic iron species is a major therapeutic goal.

General significance: The NTBI approach represents an important mechanistic concept for explaining cellular iron excess and toxicity and provides new important biochemical diagnostic tools. This article is part of a Special Issue entitled Transferrins: Molecular mechanisms of iron transport and disorders.

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1. Introduction, definition and nature of non-transferrin bound iron

Free, unbound iron is incompatible with either plasma iron transport (it would precipitate) or with intracytosolic iron trafficking (it would damage the cellular environment). Therefore, iron must be bound with appropriate ligands.

Iron in the circulation exists in complex with three major proteins, transferrin, heme, and ferritin. Transferrin (TF), produced by the hepatocytes, is a glycoprotein consisting of a single polypeptide chain with two iron-binding sites (the two sites, A and B, each binds one atom of ferric iron), and of two branched carbohydrate chains (glycans). It transports iron within the plasma and delivers iron to cells, especially to bone marrow where iron contributes to the production of new red blood cells. Transferrin exists as four molecular forms – apotransferrin, monoferric A transferrin, monoferric B transferrin, and diferric transferrin – but all transferrin bound iron can be considered physiologically as a single homogeneous pool. Mean plasma iron and transferrin concentrations are 20 μmol/L and 30 μmol/L, respectively, corresponding to a transferrin saturation of approximately 30%. Iron in complex with heme is transported as hemoglobin bound to haptoglobin and also as heme bound to hemopexin. Ferritin iron represents a minute amount of circulating iron since the iron content of plasma ferritin is low. Normal plasma ferritin levels are less than 300 μg/L in men and less than 200 μg/L in women. Besides these plasma iron forms, another iron species, termed non-transferrin bound iron (NTBI), originally identified by Hershko et al. [1], is thought to play a major role in various pathological conditions that are dominated by iron overload. NTBI corresponds to iron which is not only unbound to transferrin but also does not correspond to heme or ferritin iron. Therefore, strictly speaking, the term NTBI, classically used, is not fully appropriate.

Within the cell, iron can exist in several forms. Iron delivered to the cells by plasma, via the transferrin receptor 1 pathway, enters the cell through endocytotic vesicles. This population of vesicular iron resides in these structures before being released into the cellular cytosol. Another pool of intracellular iron is storage iron. Ferritin-bound iron represents the major form of storage iron, with each molecule of ferritin being capable of storing up to 4500 iron atoms. Hemosiderin corresponds to a degraded form of ferritin iron. Iron within the cell can also be associated with proteins as prosthetic groups and participates in multiple biological reactions. This functional iron...
pool is exemplified by proteins that catalyze reactions in the respiratory electron transfer chain, DNA synthesis, and detoxification processes. Another form of intracellular iron is the transit iron pool. First proposed by Jacobs A. [2], the transit iron pool is also called the labile iron pool (LIP) [3]. It corresponds to the iron species exerting a pivotal role between the vesicular, storage, and functional iron compartments. It is not linked to the major iron storage protein ferritin – which exerts, intracellularly, the same type of protecting effect as transferrin within the plasma – and can, therefore, be considered as a kind of "intracellular NTBI" form.

The topic of the nature of NTBI in plasma has been recently reviewed [4]. Computer simulations of metal iron equilibria in bio-fluids [5] have provided data suggesting that the main form of NTBI could be iron(III) citrate. Nuclear magnetic resonance analyses [6] of plasma samples of patients with hemochromatosis indicate that both citrate and acetate could be involved. The complex formed by the interaction between these anions and ferric iron might be monomeric or oligomeric, with the latter form being consistent with the slow rate of exchange between iron(III) citrate and apotransferrin. Albumin may also bind iron. Albumin, which is present in high concentration in the plasma (close to 40 g/L), contains many negatively charged sites on its surface suitable for iron(III) binding. This binding has been demonstrated in the presence or absence of citrate anions and even when plasma transferrin is not fully saturated [7]. The exact nature of plasma NTBI remains to be determined. It is likely that different forms co-exist and that plasma NTBI species vary according to the degree of iron excess, to its duration, and to the etiology. Thus, in thalassemic patients, in vitro experiments indicated that NTBI is unlikely to be bound to albumin to a significant extent and is only loosely bound to a ternary iron–citrate–albumin complex [8]. Another special form of NTBI in plasma has been identified and is defined by its ability to engage in redox cycling. This form has been termed labile plasma iron (LPI) [9,10].

With respect to cellular NTBI [11], the nature of the iron trafficking within the cytosol – which corresponds to the transit iron pool or labile iron pool – remains poorly defined. To avoid the production of reactive oxygen species (ROS), iron should be in an appropriate sequestered form. This might correspond to iron loosely bound to cellular proteins or to low-molecular weight ligands such as citrate. Glutathione could also be a key component of the cytoplasmic iron pool [12]. Like copper [13], iron could also be linked to a cytosolic chaperone. A chaperone candidate is PCP1 (human poly(rC)-binding protein 1) which is able to deliver iron to ferritin in yeast (Saccharomyces cerevisiae) and also in mammalian cell lines [14].

2. Cellular uptake and release of NTBI

NTBI is avidly taken up by several tissues, which supports the role of NTBI as an important source of organ iron deposition (Figs. 1 and 2).

2.1. NTBI and the liver

NTBI is efficiently taken up by the liver, as first suggested by the observation that in rats in which acute saturation of plasma transferrin iron-binding capacity had been induced, a large fraction of intestinally absorbed iron was deposited in the liver [15]. Using the single-pass perfused rat liver, iron uptake as NTBI ranged from 58% to 75% as opposed to less than 1% with transferrin-bound iron [16]. The intravenous injection of 59Fe in rats or mice whose total transferrin iron-binding capacity had been induced, a large fraction of intestinally absorbed radioactive iron was almost completely deposited in the liver, as less than 1% was deposited in the erythrocytes [17,18]. The newly absorbed radioactive iron was almost completely deposited in the liver while less than 1% was deposited in the erythrocytes [17,18]. NTBI mainly targets the hepatocytes. In the perfused rat liver [16], ferrous iron accumulated as electron dense particles compatible with ferritin cores within the hepatocytes with preferential localization in the lysosomes. Rat hepatocytes in primary cultures have been shown to exert a high capacity for taking up NTBI as the Fe-citrate form [19]. Evidence for a low Km transporter for NTBI has been reported in isolated rat hepatocytes [20]. In hypotransferrinemic mice, electron microscopy and laser probe mass analysis showed preferential iron deposition within the hepatocytes, in the form of ferritin particles and clusters as well as ferritin and hemosiderin in lysosomes [21]. NTBI uptake by the liver is not down-regulated by hepatic iron excess [19,22], in contrast with transferrin iron uptake which is down-regulated when iron stores increase in the cells, due to the IRE/IRP system which regulates transferrin receptor expression. Using transformed cell lines (fibroblasts [23], HepG2 cells [24]) intracellular iron loading led to an increased NTBI uptake. In cultured rat hepatocytes and hepatoma cells, the iron chelators desferrioxamine and deferoxamine have been reported to inhibit NTBI uptake essentially through their binding to NTBI during the uptake phase rather than through mobilization of iron which has been stored as NTBI [25].

2.2. NTBI and other organs

In the exocrine pancreas of the hypotransferrinemic mouse model [26], the organ is particularly overloaded with iron; the involvement of centro-acinar and intercalated duct cells was massive and generalized, whereas macrophages contained isolated siderosomes only. In the islets, β cells rarely showed siderosomes and no ferritin particles were seen in the cytosol [21].

In the heart, cardiomyocytes of hypotransferrinemic mice were significantly iron overloaded [21] and in heart cell cultures, the rate of NTBI uptake is more than 300-fold greater than that of transferrin iron [27]: moreover, it is increased by high tissue iron content [24]. 59Fe uptake by the brain was 80–85% times greater in hypotransferrinemic mice than in normal controls [28]. The absence of abnormal iron distribution [29] suggests that there is a NTBI delivery system within the brain.

With respect to erythroid cells, rabbit reticulocytes have been shown to incorporate NTBI [30,31]. In the Belgrade rats (a model of microcytic anemia due to mutations of the Divalent Metal Transporter 1 (DMT1)), two types of NTBI uptake by the erythroid cells were observed [32]: i) one type requiring DMT1 since it was abolished in the Belgrade rats. It corresponds to a high affinity process providing iron for heme synthesis (and concerns reticulocytes but not the erythrocytes); ii) the second type does not require DMT1 and is operative in erythrocytes but not in reticulocytes. Using flow cytometry techniques, Prus et al. [33] found that both the transferrin receptor1 (TfR1)-deficient mature red blood cells and their transferrin receptor-containing precursors at all stages of maturation can take up NTBI that accumulates as redox-active labile iron and generates reactive oxygen species. Such a mechanism could account for the ineffective erythropoiesis of developing precursors in the bone marrow and for the shortening of the lifespan of mature erythrocytes in the circulation.

2.3. Molecular aspects of NTBI iron uptake

The uptake of iron in the NTBI pool requires carrier molecules. The action of DMT1 has been well demonstrated at the enterocyte level with the absorption of dietary iron [34] whereas at the hepatic level, it appears to be dispensable since mice which lack DMT1 are still able to accumulate hepatic iron [35]. The role of DMT1 for NTBI uptake has been further illustrated by the use of normal hepatocytes [36], hepatoma (HFL) cells, and hepatic cells from Hfe knock-out mice that exhibit upregulation of DMT1 expression [37].

The Zrt–Irt-like protein 14 (ZIP14) (SLC39A14) is a zinc transporter and represents the other major carrier involved in NTBI uptake by hepatocytes [38]. Recent data indicate that Zip14 also promotes cellular assimilation of iron from transferrin [39].

Please cite this article as: P. Brisset, et al., Non-transferrin bound iron: A key role in iron overload and iron toxicity, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagen.2011.07.014
L-type voltage-dependent calcium channels may facilitate NTBI uptake by cardiac myocytes as suggested by the inhibitory effect of the corresponding channel blockers [40]. Mechanistically, whether L-type calcium channel blockers act through increasing DMT1-mediated cellular iron transport [41] remains under debate [42].

Lipocalin2, another potential candidate for NTBI uptake [43], is not essential for iron delivery to hepatocytes in the context of HFE deficiency [44].

The transferrin receptor 2 (TfR2) is essentially expressed in the liver and has been shown experimentally to mediate both transferrin-bound and NTBI uptake. However, the cellular model used in this study (CHO cells) did not express hepcidin, the hormone that regulates iron homeostasis [45]. In fact, its role in transferrin–iron and/or NTBI uptake is very limited since i) its affinity for diferric transferrin is 25–30 fold lower than TfR1, and ii) hemochromatosis develops in humans with TfR2 mutations [46] and in mice in which the TfR2 gene has been deleted [47]. TfR2 may act essentially as an iron sensor participating in hepcidin regulation, and plasma NTBI levels could be its main signal.

HFE can lower NTBI uptake in isolated primary mouse hepatocytes [37] and in CHO cells [48]. Using HepG2 cells, Gao et al. [49] showed that HFE decreases the stability of Zip14 and reduces NTBI (and also transferrin bound iron) uptake.

Scara5 is a recently identified ferritin receptor mediating NTBI delivery in the developing kidney [50]. Whether this pathway may be relevant in other organs, especially the liver, remains to be explored.

It has been reported that NTBI itself can reduce transferrin–iron uptake [51,52], suggesting that transferrin–iron can be delivered to cells without endocytosis, and possibly by an uptake process involving a cell surface ferric iron reductase [53].

Whatever the transporter involved in NTBI uptake, a pre-requisite for this uptake is a reduction process since iron cannot cross the membranes in the ferric (oxidized) state [54]. Ferric reductases have been first described in yeast (S. cerevisiae) [55]. In mammalian cells, duodenal cytochrome B (DCYTB) (CYBRD1) is a cytochrome B reductase which plays an important role at the level of the brush border membrane of the enterocytes [56], although it seems dispensable since mice lacking the DCYTB gene do not develop an overt phenotype [57]. Similarly, the release of iron from endosomes requires a reductase, STEAP3 [58].

2.4. Cellular sources of plasma NTBI

The source of plasma NTBI is not fully understood. Whether NTBI appears primarily after cellular iron egress or secondarily due to the equilibrium between iron linked to transferrin and the potential carriers of NTBI remains to be determined.

During cellular iron egress, ferroportin (FPN) (SLC40A1) is the only protein carrier yet known [59–61]. Since hepcidin, the iron hormone, acts on ferroportin to control the egress of iron from cells into plasma [62], it is clear that the degree of hepcidin synthesis (essentially by the hepatocytes) is a primary event for determining plasma iron concentration, and, in turn, transferrin saturation and the possible appearance of NTBI (low plasma hepcidin levels leading to high transferrin saturation and to plasma NTBI).

In a reverse of the mechanism that occurs for cellular iron uptake, an oxidation step is necessary after iron is released from the transmembrane channel into the blood stream to allow the uptake of iron as the ferric form by transferrin. Two main oxidases are involved: at the plasma enterocyte level, hephaestin (Hp) (HEPH) [63–65] and, at the plasma macrophage level, ceruloplasmin (Cp). It is noteworthy that, during aceruloplasminemia, in which the plasma iron content is very low due to absence of ferroxidase activity, NTBI is not found in plasma [66]. Therefore, the chemical equilibrium between transferrin

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Please cite this article as: P. Brissot, et al., Non-transferrin bound iron: A key role in iron overload and iron toxicity, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagen.2011.07.014
and other carriers of NTBI, especially those of low molecular weight is likely important. At the hepatocytic level, NTBI which originates from plasma, is also partly eliminated through the biliary pathway [67]. In the HFE knock-out mouse, iron release by hepatocytes is not modified by an increase in NTBI uptake [68].

3. Determination of NTBI

3.1. Determination of plasma NTBI

Several assays for the detection of NTBI have been proposed and they take into account the technical difficulties related to determination of heterogeneous chemical forms of circulating NTBI.

The chelation–ultrafiltration–detection approaches are based on the prior mobilization of serum NTBI by agents such as ethylenediaminetetraacetic acid EDTA [1], oxalate [69], and nitrilotriacetate (NTA) [70]. After ultrafiltration, which separates the chelated NTBI from transferrin iron, NTBI detection is performed according to colorimetric methods [71], HPLC techniques [70,72–74], or atomic absorption spectrometry [75]. When transferrin is only partially iron saturated, the addition of a cobalt salt blocks the free iron binding sites in order to avoid donation of iron from NTA onto the vacant sites of transferrin, thereby incurring an underestimation of NTBI values [76,77]. Use of an optimal concentration of the chelator NTA has been reported to improve the method [78].

Detection methods involving bleomycin are based upon the ability of the antitumor antibiotic to degrade DNA via a free radical reaction in the presence of ferrous ions. These ferrous ions are produced through the addition of a reducing agent (such as ascorbate) to the serum, and the free radical reaction is detected by the production of thiobarbituric acid-reactive substances which include malondialdehyde [79]. However, some of these products can be generated during the high temperature phase of the method and therefore correspond to artifacts [80]. Applying the ethidium-binding assay of DNA damage to the measurement of bleomycin-detectable iron has been reported to improve the reliability of the method [81].

Several NTBI detection methods involve the use of fluorescent probes. Breuer et al. [82] have developed a method for measuring the desferrioxamine-chelatable iron (DCI) component of serum NTBI. Serum DCI is measured with the probe fluorescein-desferrioxamine whose fluorescence is, in turn, stoichiometrically quenched by iron. The mobilizer-dependent chelatable iron (MDCI) assay determines the fraction of oxalate-mobilized serum iron bound by fluorescein-apotransferrin [83]. Using a fluorescent probe of the hexadentate chelator CP851, Hider et al. measured NTBI levels down to 0.1 μM with repeatable accuracy [84]. Finally, a procedure that uses fluorogenic dihydorhodamine 123 permits quantitation of the LPI. This assay is an important methodological advance since it detects the redox active component, and therefore the potentially toxic species, of plasma NTBI. Dihydrorhodamine 123 permits the monitoring of the generation of reactive radicals prompted by ascorbate but which are blocked by iron chelators [85].

An international round robin for the quantification of serum NTBI showed that NTBI values differ considerably between methods [86].

3.2. Determination of "cellular NTBI"

Fig. 2. The cellular interactions between transferrin iron (Tf-Fe) and non–transferrin bound iron (NTBI). The enhanced labile iron pool (LIP) leads to decreased expression of the Transferrin Receptor1 (TfR1) through the IRE–IRP system, but it has no effect on cellular NTBI uptake. Reductase (green) Oxidase (red). Transporter. Transferrin receptor 1 (TfR1).

The levels of the labile iron pool have been specifically assessed in living cells by a method that originally used digital fluorescence microscopy and that was subsequently adapted to use laser scanning microscopy. The procedure is based on quenching of the fluorescent transition-metal indicator Phen Green SK by intracellular chelatable iron, followed by dequenching the indicator’s fluorescence with an excess of membrane-permeable iron chelators [87]. Another noninvasive
technique based on the application of fluorescent metalosensors uses calcein [3]. With various fluorescent probes sensitive to iron or to reactive oxygen species, targeted to cytosol and/or to mitochondria, Silvartsman et al. [88] traced the ingress of labile iron into these compartments by fluorescence microscopy and quantitative fluorimetry. It was observed that: i) penetration of NTBI into the cytosol and subsequently into mitochondria occurs with a barely detectable delay, and ii) loading of the cytosol with high-affinity iron-binding chelators does not abrogate iron uptake into the mitochondria. Therefore, a fraction of NTBI acquired by cells reaches the mitochondria in a non-chelatable labile form.

In the case of experimental iron overload, Zanninelli et al. have shown that LPI increased significantly [89].

4. Role of NTBI in iron-related disorders

Several pathological conditions can contribute to increased levels of NTBI in the plasma as listed in Table 1 and discussed below.

4.1. NTBI as a source of cellular and organ iron excess

4.1.1. Genetic iron overload disorders

Prior to the identification of HFE, the overloading role of NTBI was first reported by Fawwaz et al. [90] who demonstrated, in untreated hemochromatosis, a high first-pass hepatic deposition of intestinally absorbed iron. The presence of plasma NTBI was also detected in several later studies.

Following the HFE gene discovery, it has become possible to evaluate NTBI in more precisely defined conditions of genetic iron overload [91]. In type 1 (HFE-related) hemochromatosis, NTBI has been characterized by Loréal et al. [77], who showed that NTBI levels: i) reached values as high as 5 μM and could be detected even when transferrin was not fully saturated; ii) persisted almost until completion of the phlebotomy induction phase; iii) were well correlated in a given patient with transferrin saturation through two combined mechanisms: i) increased iron release from overloaded macrophages (following the sequestration and degradation of transfused erythrocytes) and ii) increased iron release from the enterocytes (due to hepcidin deficiency, related itself to dyserythropoiesis). In contrast, NTBI/LPI seems to play no significant role in sickle cell disease [93–95].

4.1.2. Hematological iron overload disorders

Particularly high levels of NTBI and LPI have been found in beta-thalassemia major and intermedia [85,92]. This event is likely related to increased plasma transferrin saturation through two combined mechanisms: i) increased iron release from overloaded macrophages (following the sequestration and degradation of transfused erythrocytes) and ii) increased iron release from the enterocytes (due to hepcidin deficiency, related itself to dyserythropoiesis). In contrast, NTBI/LPI seems to play no significant role in sickle cell disease [93–95].

4.1.3. Other disorders

Diseases not primarily related to iron overload but which can involve disturbances of iron metabolism can contribute to NTBI appearance.

In liver diseases, NTBI is likely to be observed through hepcidin deficiency related either to etiology (especially alcoholism [96]) or to the severity of the liver dysfunction (acute hepatocellular failure in fulminant hepatitis or chronic hepatocellular failure in severe cirrhosis [97]). In the latter situation, the main factor generating NTBI is low transferrin concentration due to impaired hepatic synthesis. As a clinical illustration, De Feo et al. [98] reported that NTBI was found in more than 83% of a cohort of active chronic alcoholics and that patients with cirrhosis had significantly higher NTBI than observed in control patients. This same study reported the presence of NTBI in patients with transferrin saturation lower than 45%, suggesting that mechanisms distinct from the simple chemical equilibrium between transferrin and low molecular weight carriers of iron are involved.

NTBI levels have also been examined in a number of other disorders. In cases of hematological malignancies, NTBI is frequently present during myeloablative therapy and stem cell transplantation [99,100,101]. In cases of hematological malignancies, NTBI is frequently present during myeloablative therapy and stem cell transplantation [99,100,101]. In these cases, in vitro administration of exogenous apotransferrin had a beneficial effect against infections by S. epidermidis [102].

NTBI has also been reported in diabetes [103,104] but not in all cases [105], in the myelodysplastic syndrome [106,107], and in end-stage renal disease [108,109] with enhanced S. aureus growth [110]. Regarding the debated issue of the relationship between iron and atherosclerosis, it should be noted that no increased risk of coronary heart disease in relation to NTBI levels has been found in postmenopausal women [111]. Likewise, no correlation was found between serum NTBI levels and measures of in vivo LDL oxidation despite a correlation between NTBI and sICAM1 (soluble intercellular adhesion molecule) [112].

4.2. NTBI as a factor in cellular and organ toxicity

Iron is known to promote ROS via the Haber–Weiss reaction [113]. ROS, in turn, are prone to generate increased lipid peroxidation [114]. Iron-induced oxidative damage has been shown in plasma membrane and in different subcellular targets including rat hepatocytes [115,116], hepatic lysosomal membranes [117], hepatic [118,119] and cardiac mitochondria [120], as well as in the nuclei [121,122]. Moreover, at the hepatocyte level, NTBI may exert cellular toxicity by increasing cellular iron content through both increased iron entry and decreased iron release via the biliary tract [67]. Enterohelial recycling has been demonstrated in rats [123], however the pathophysiological importance of this phenomenon is uncertain.

Emerging data have provided arguments favoring the damaging role of NTBI in various clinical situations. NTBI has been reported to be correlated with both hepatic and cardiac damage in thalassemia [125]. A correlation between plasma LPI and alanine aminotransferase (ALT) levels has been shown in type 1 hemochromatosis [124].

Regarding therapeutic aspects, phlebotomies are known to represent the mainstay for eliminating iron excess in hemochromatosis. However, in so far as transferrin saturation remains very high during the whole induction phase of iron depletion (it drops to

Table 1

Main pathological situations leading to plasma NTBI.

<table>
<thead>
<tr>
<th>Diseases with major iron overload</th>
<th>Other diseases</th>
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<tbody>
<tr>
<td>Beta-Thalassemia*</td>
<td>Alcoholic liver disease</td>
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<tr>
<td>Myelodysplasia*</td>
<td>Acute or chronic hepatic failure</td>
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<tr>
<td>Hemochromatosisb</td>
<td>Diabetes</td>
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<tr>
<td>HFE-related (type 1)</td>
<td>End-stage renal failure</td>
</tr>
<tr>
<td>Non-HFE-related (types 2 and 3)</td>
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* Major role of transfusions.

b Major role of hepcidin deficiency.
normal levels only at the very end of this phase), it is likely that the iron recycling produced by the venesection could contribute to a transient increase in NTBI levels, representing therefore a potential undesirable effect. Therefore, ongoing efforts are required to develop improved therapeutic strategies and to discover the best protocols that will permit the elimination of plasma NTBI throughout the day. Associating oral chelation may be an interesting approach, in light of the results observed in iron overload of hematological origin [126].

5. Conclusions
Non-transferrin bound iron corresponds mainly to circulating iron species which appear when transferrin saturation is increased. Elevated levels of NTBI are potentially responsible for cellular damage both at the cellular surface and at the intracellular level. The concept of NTBI has been extended to include special forms of trafficking iron, either in the plasma or within the cellular milieu, which are not linked with the major carrier and storage proteins – transferrin and ferritin, respectively – and therefore present a high potential for toxicity. Avoiding NTBI formation is therefore an important therapeutic goal in all pathological conditions, particularly in iron overload disorders, that can lead to generation of this deleterious form of iron.

References


