Nontransferrin-bound iron and labile plasma iron in relation to iron toxicity
unraveling the confusion

NTBI **LPI**

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Disclosure of Interests

- Consultant for Aferrix and Hinoman, Tel Aviv, Israel
- Research Contract: Shire, UK
- ESB: Novartis, N.J., USA
- Honoraria: Apopharma, Canada

LABILE IRON (LI) in biological systems (see references)

Is present in cells (rarely in internal fluids) and is comprised of Fe (II & III) forms that are:

a. chemically (redox) active

- **inter-convertible Fe(II↔III) by bio-redox active agents.**
- **capable of promoting formation of reactive O (RO) species (ROS) by reacting with O_2 or with RO intermediates (ROI) (e.g. H_2O_2 and $O_2^{\cdot}$-, byproducts of respiration and other O_2-consuming reactions)**

b. exchangeable

between *(bio)*ligands and/or *(bio)*metals and also *chelatable (!).*

Labile cell iron (LCI) is an integral component of living cells

- **LCI is detected in intact cells [0.1-1.5 μM] distributed in the cytosol, mitochondria and lysosomes.**
- **LCI is comprised of complexes of iron with nucleotides, gluthathione and carboxylates (di-OH benzoates?).**
- **LCI levels are determined by the redox potential (NADPH, NADPH, GSH) of the respective cell compartments**
- **LCI cytosolic (LCIc) is at the cross-roads of cell iron management and is maintained homeostatically (so as to meet metabolic needs while minimizing risks of involvement in noxious radical formation).**

LCI is pathophysiologically relevant:

- **LCI promotes ROS formation when it raises to "relatively high" levels, defined as levels that surpass cell innate capacity to produce sufficient ferritin (to absorb excess iron) or antioxidants that counteract formation of reactive O species (ROS).**
- **LCI raises to high levels due to either:**

  a. cell Fe maldistribution that results in regional siderosis (systemic or regional), as found in various acquired and inherited metabolic iron disorders or

  b. infiltration by labile and membrane permeant forms of iron that appear in iron overloaded plasma (defined as LPI= labile plasma iron, a major component of NTBI=non-transferrin-bound iron) such as in systemic siderosis (hemochromatosis, primary or transfusional).

- **LPI infiltrates cells "opportunistically" (*via resident cell membrane transporters or bulk endocytosis*).**

LCI and LPI are pharmacologically relevant:

- **As, "excessively high" LCI and LPI are potentially toxic chemical entities that are also chelatable, they are perceived as direct pharmacological targets of chelation.**
- **Polymeric iron forms (PIF) given iv "are designed" to have minimal labile iron and generate no LPI.**

LABILE CELL IRON (LCI)

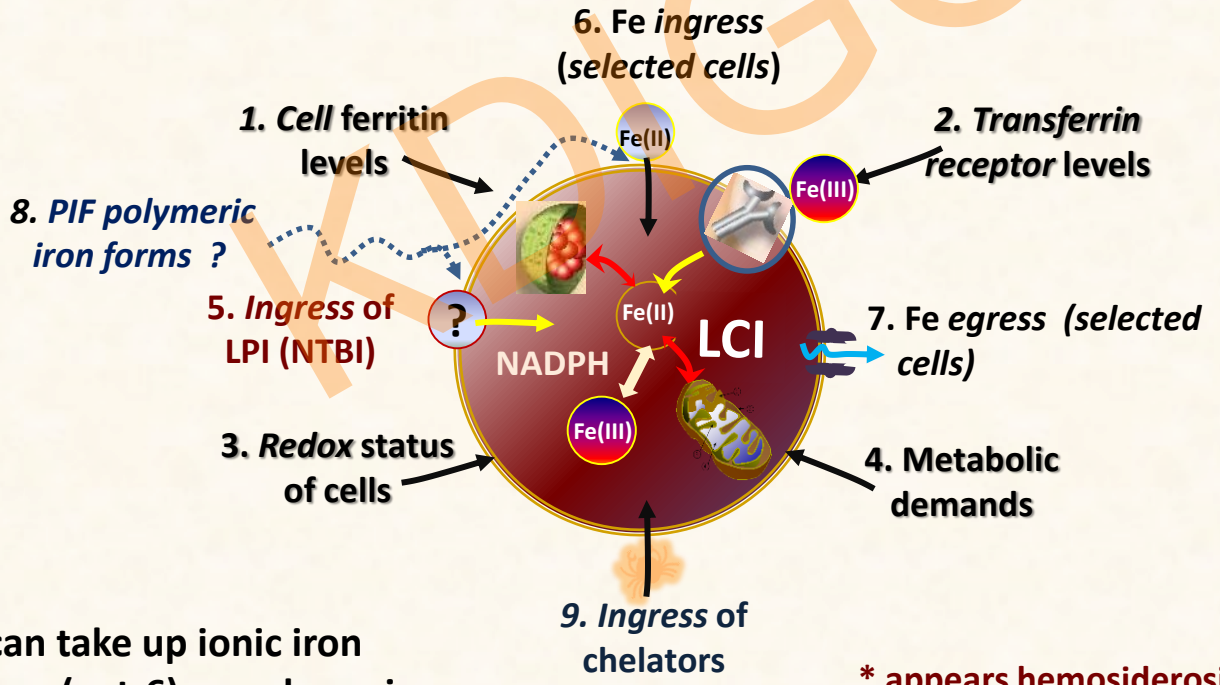
factors that affect LCI levels

Physiology

- Cell iron levels are coordinately regulated by transferrin receptors and ferritin (1&2), whose expression is dictated by LCI levels and
- modulated by cell redox potential (3) and cell metabolic needs (4)

Pathology (iron overload) :

- *Systemic*- accumulation is caused by cell exposure to LPI (NTBI) (5) *
- *Regional*- accumulation (primarily in mitochondria) is caused by impaired capacity to utilize mitochondrial iron**.



Selected cells can take up ionic iron from the medium (gut-6) or polymeric iron (PIF-8) from plasma or extrude iron into plasma (gut & macrophages-7)

* appears hemosiderosis (hereditary, transfusional, chemotherapy) when TSA>70%

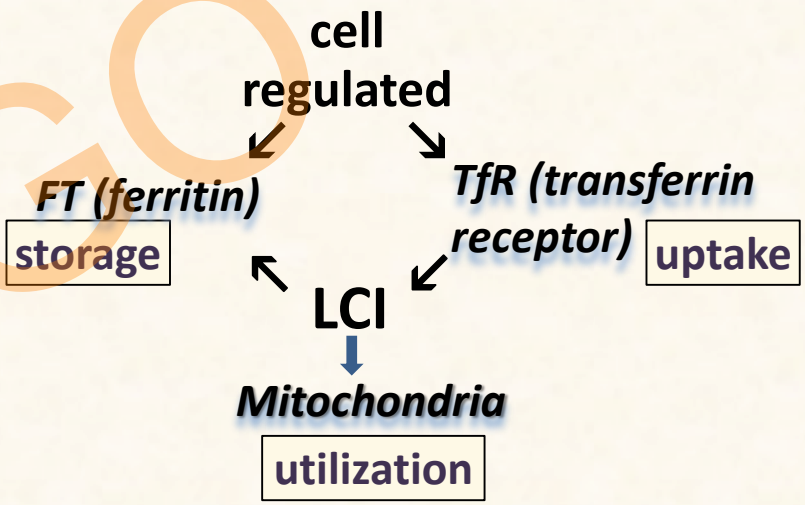
** e.g. Fr. Ataxia, various sideroblastic anemia)

LABILE CELL IRON (LCI)

physiology

LCI is a fraction of the cell Fe pool that is:

- ***redox active*** **[Fe(II) ↹ Fe(III)]**
- ***exchangeable/chelatable***
- ***transitory*** **and** ***metabolically active***
- ***regulated*****: uptake/storage/utilization**
- ***measurable: represents*** **<1% of cell Fe (which is mostly protein-associated via prosthetic groups such as heme or FeS clusters**



LCI composition is variable:

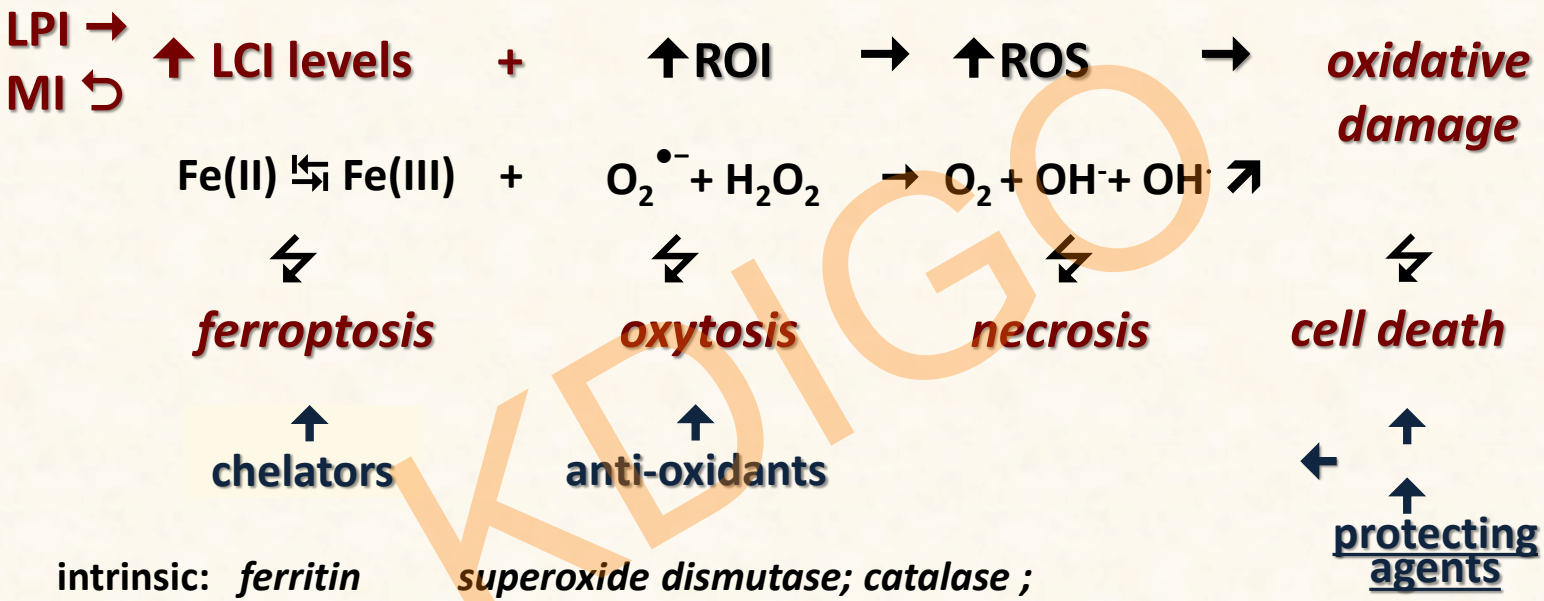
depends on the redox cell status, exposure to external Fe sources, their concentration and time of exposure. Potential LCI complexes:

- **nucleotides**
- **glutathione**
- **di(OH) benzoates (?)**

LCI **has been referred as a Loch Ness monster due to: a. the inability to capture it in situ and b. the propensity for Fe(II)↔ Fe(III) conversion.**

LABILE CELL IRON (LCI)

overload, toxicity and cell death types



intrinsic: *ferritin*
extrinsic: *DFP, DFX, "DFO"*

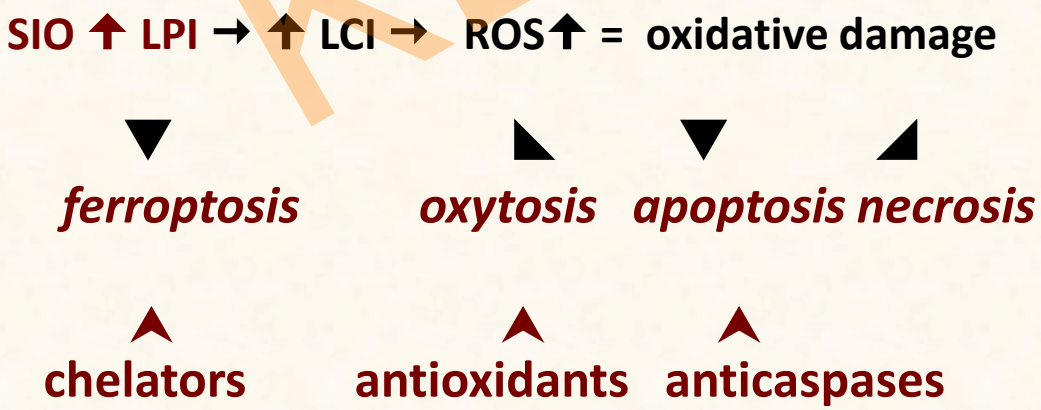
superoxide dismutase; catalase ; glutathione (peroxidase); bilirubin, urate, GSH, ascorbate, vitamin E

MI= maldistributed iron

IRON TOXICITY IN SYSTEMIC IO DISEASES

Pathology (hemosiderosis = systemic iron overload SIO)

- **Indicators of iron overload (IO) ,such as T2* MRI or Perl's stain, reflect iron agglomerates (ferritin or hemosiderin) that are chemically inert.**
- **IO can be considered pathological when supported with evidence (biochemical, histological and functional) of oxidative damage**
- **The involvement of labile iron in toxic IO is demonstrated by the protective effect of permeable iron chelators.**



LABILE IRON IN BIOLOGICAL SYSTEMS

SELECTED REFERENCES (from Cabantchik, Z.I et al.) and their links

- Cabantchik, Z.I. (2014). Labile iron in cells and body fluids. Physiology, Pathology& Pharmacology. *Front. Pharmacol* 4:1 doi: 10.3389/fphar.2014.0004. Review o/l. : http://journal.frontiersin.org/Journal/10.3389/fphar.2014.00045/full

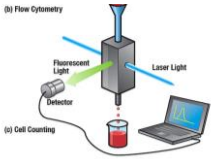
- Sohn, Y.S., Ghoti, H., Breuer, W., Rachmilewitz, E.A., Attar, S, Weiss, G. and Cabantchik, Z.I. (2012) The role of endocytic pathways in cellular uptake of plasma non-transferrin iron. *Haematologica* 97:670-678

- Breuer, W., Shvartsman, M., Cabantchik, Z.I. (2007) Intracellular labile iron. A review. *Int J Biochem Cell Biol*. 40: 350-354. http://www.ncbi.nlm.nih.gov/pubmed/17451993.
- Esposito, B.P, Breuer, V.W., Slotki, I.N. and Cabantchik, Z.I. (2002). Labile iron in parenteral iron formulations and its potential for generating plasma non-transferrin bound iron (NTBI) in dialysis patients. *Eur. J. Clin. Inv.* 1:42-9.* http://www.ncbi.nlm.nih.gov/pubmed/11886431?dopt=Abstract

- Breuer, W., Ronson, A, Slotki, I.N., Abramov, A., and Cabantchik, Z.I. (2000). The assessment of serum non-transferrin-bound iron in chelation therapy and iron supplementation.. *Blood* 95:2975-2982. http://www.ncbi.nlm.nih.gov/pubmed/10779448

***note:**

"non-transferrin-bound iron" should have been labelled "labile plasma iron"



LABILE CELL IRON (LCI)

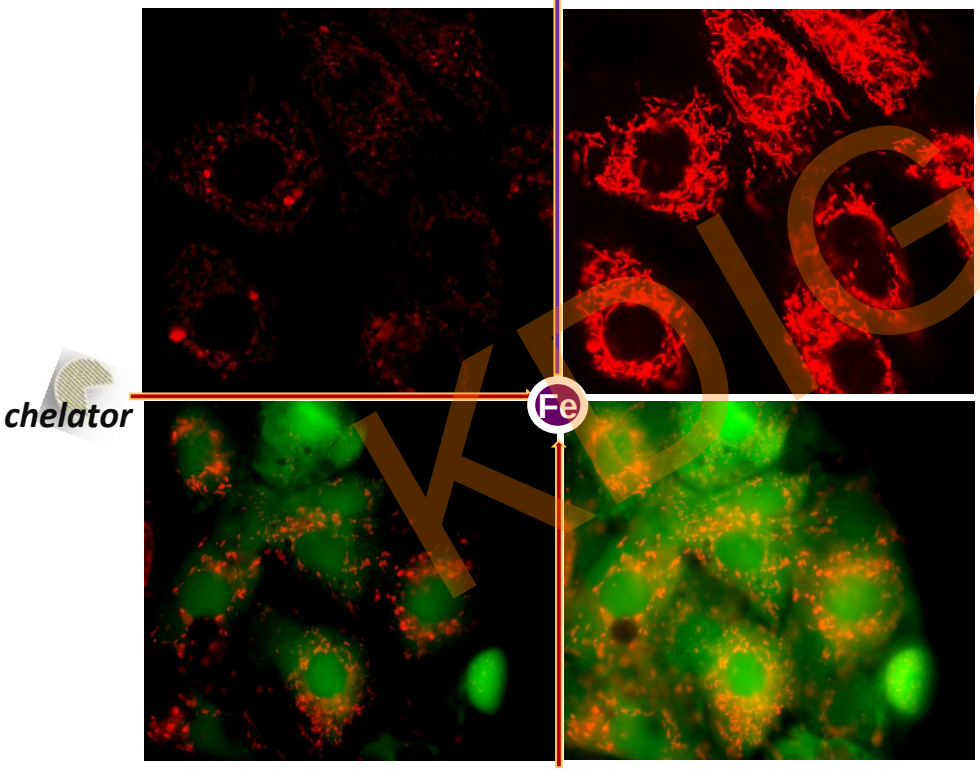
Measurements based on fluorescence metal and ROS sensors

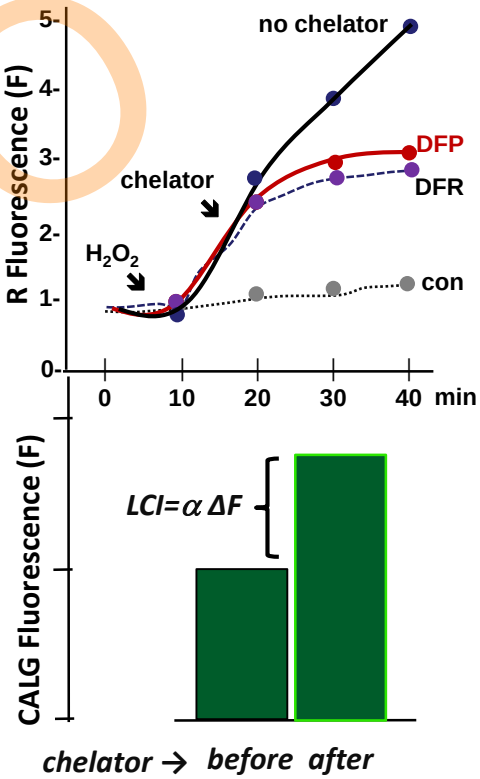
LCI as redox-active and chelatable iron

non-fluorescent oxidizable precursor dihydrorhodamine DHR

DHR $\xrightarrow{H_2O_2}$ R

DHR oxidizes to fluorescent R by ROS generated from LCI prompted with H_2O_2





quenched CALG-Fe CALcein-Green

Fe +chelator *fluorescent CALG*

The recovery of CALG fluorescence ΔF elicited by a chelator reveals CAL-Fe, which is ≅ LCI

LCI as directly chelatable iron

LABILE PLASMA IRON AS SOURCE OF LABILE CELL IRON

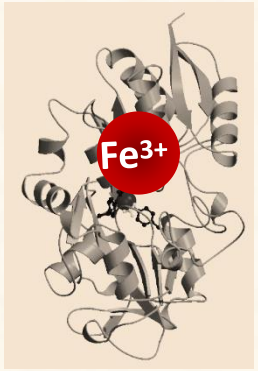
- **The term LPI, the extracellular counterpart of LCI, was introduced in order to define the component of plasma NTBI that is redox-active, permeant to cells and chelatable. (Breuer, Hershko and Cabantchik 2000).**
- **The original use of the term NTBI (Hershko et al 1978) was to denote plasma iron that is not bound to transferrin and is extractable with mild metal complexing agents and filterable. The term was problematic, since it defined "something by what it is not" (an apophasis). Thus, *stricto senso,* any Fe form in plasma that is not transferrin-bound (TBI), qualifies as NTBI, irrespective of its potential toxicity (e.g. Fe-chelates, PIFs, ferritin).**
- **LPI has been detected only in pathological conditions in plasma or serum from patients with TSAT>70%.**

Measuring plasma NTBI (total)

plasma

Hershko el 1978 ; Hider, Porter et al 01
extraction & filtration

transferrin

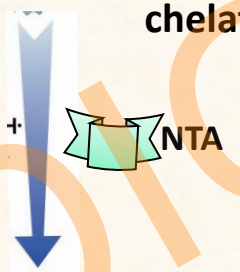


40 µM

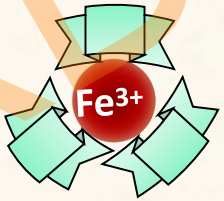
NTBI, mostly protein adsorbed

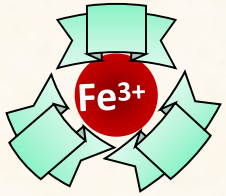
2 µM

1. Extraction via "mild" chelation (80 mM NTA)



2. filtration





3. Detection with sensor (± HPLC)

accurate, sensitive, reproducible.

laborious; might mobilize Fe from Tf , Fe-chelates and PIF

Measuring LPI, the labile/catalytic component of plasma NTBI

catalytic iron: *Gutteridge and Haliwell 80's; Bonsdorff 02'.*

TBI

40 µM

NTBI

2 µM

LPI: labile plasma iron
Esposito et al 03

ascorbate reduces LPI to Fe(II)

that binds to bleomycin

and promts generation of oxidative radicals

bleo

no ← Fe chelator → yes

problematic!

blocks

that oxidize DNA or deoxyribose

color product

detection with TBA Thiobarbituric acid

that redox-cycles and reacts with a probe that transforms from

DHR

non-fluorescent substrate DHR to fluorescent product R

R

High throughput fluorescence assay for any LI-containing fluid

Estimates of LPI and NTBI in patients with "iron overload“

Random selection from 18K samples of patients treated or not with chelators

Patient group (% is of patients in each category)	LPI	NTBI
	mM	mM
Thalassaemia (intermed. 30%, major 50%)	0.5–10	0.5 to >10
Hereditary haemochromatosis 15%	0.2–2.0	0.1–5.0
(6-10 weeks after last phlebotomy)	0.2–0.6	0.1–1.0
MDS (transfused) ~ 30%	0.5–2.5	0.1–3
• Chronic (advanced) diabetes ~ 35%	0.2–2.2	
• Random controls < 1%	0.2–0.6	-0.2–1.0

No detectable levels (<0.2 μM) of LPI in normal individuals. Persistent LPI values > 0.2 μM at trough plasma concentrations of chelators are considered significant and indicative of either impending or overt systemic iron overload

POTENTIAL IRON TOXICITY IN THE TREATMENT OF ANEMIA WITH PIF (polymeric iv iron formulations)

The infusion of 100-200 mg PIF ("NTBI") iron raises plasma iron of an anemic patient with e.g. an UIBC < 30 μM (TIBC ≅ 40 μM) to 0.45-0.9 mM (i.e. >500 fold higher than NTBI in hemosiderosis!)

safety issues to be considered regarding possible formation of LPI at the site of PIF iv administration

↓

PIF iv administration

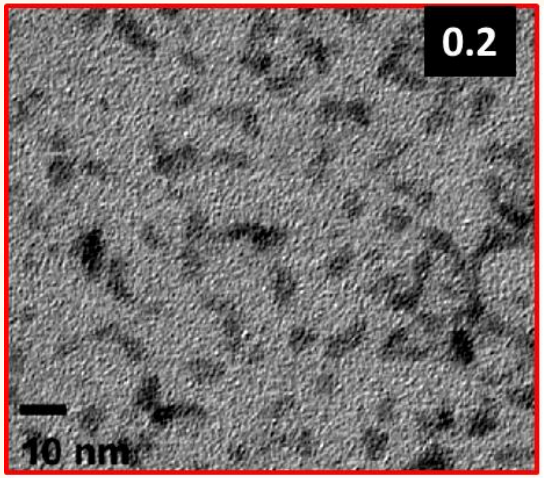
safety issues

a. a contamination of PIF itself with labile iron (LI) is potentially toxic in plasma if there is insufficient UIBC at the site of administration (as LPI is a chemically reactive species it can lead to oxidation of serum components and render them immune-compromising
b. PIF LI might increase with time on the shelf and in circulation
c. potential sensitivity of patients with high levels of plasma oxidants (e.g. chronic diabetes, inflammation)
d. long-term retention of PIF accumulated in the spleen and liver and possible spillover to pancreas

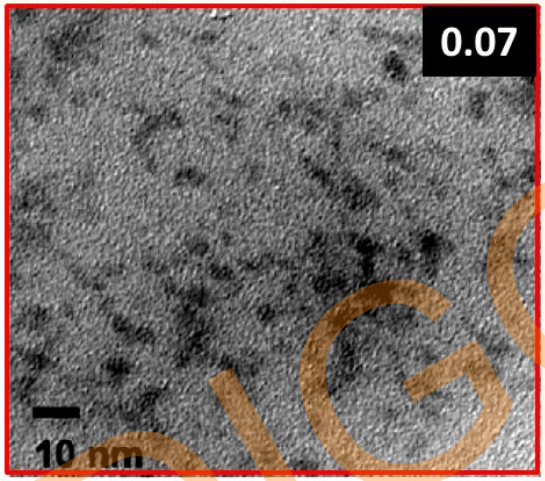
TEM 1mg/ml Fe, nanoparticles

relatively low % of dialyzable (not necessarily labile) iron in various PIFs

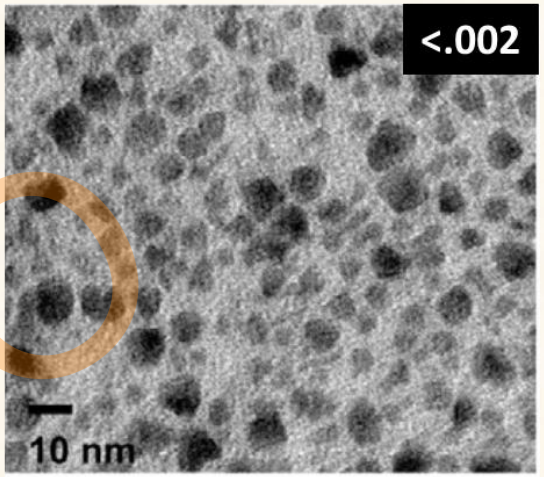
Jahn et al 2011



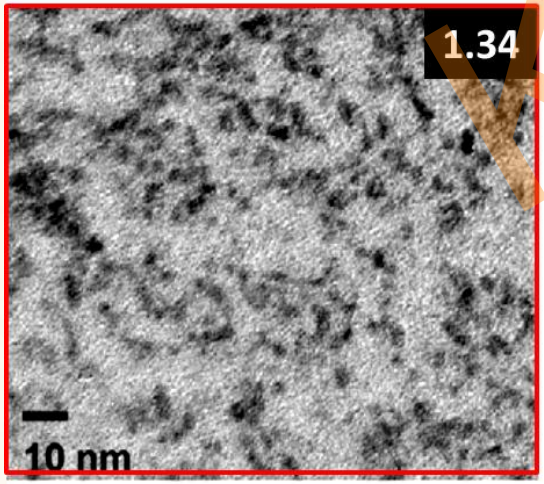
LMW iron dextran



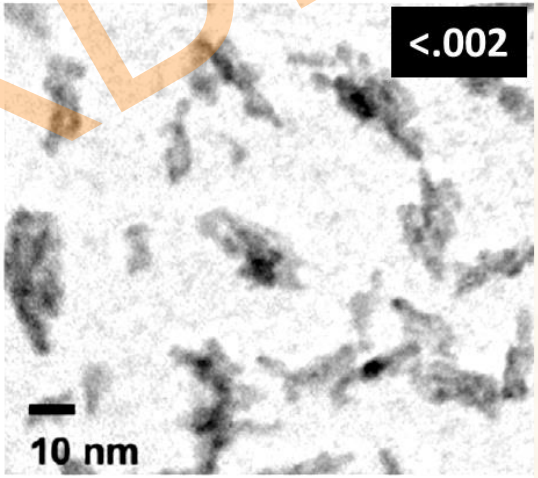
iron sucrose



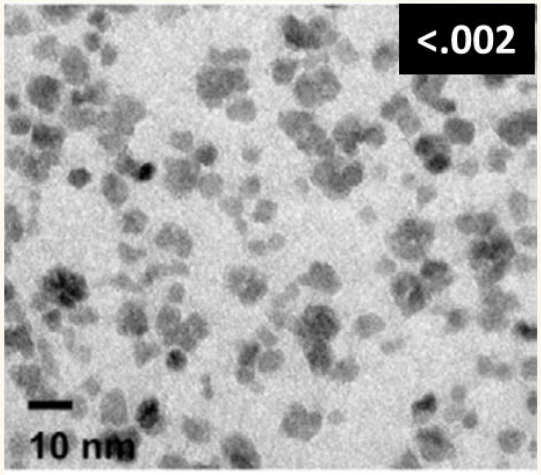
iron isomaltoside



sodium ferric gluconate



iron carboxymaltose



ferumoxytol

Labile iron in PIFs and in plasma of patients infused with PIFs

The assessment of non-transferrin bound iron (NTBI) in iron chelation therapy and iron supplementation. *Blood. 2000 May 1;95(9):2975-82.*
Breuer W, Ronson A, Slotki IN, Abramov A, Hershko C, Cabantchik ZI.

Labile plasma iron in parenteral iron formulations and its potential for generating non-transferrin iron (NTBI) in dialysis patients
European Journal of Clinical Investigation (2002) **32** (Suppl. 1), 42–49

B. P. Espósito, W. Breuer, I. Slotki* and Z. I. Cabantchik

N=71 dialysis patients:
LPI: normal levels (< 0.2 μM) in 80 % (measured 1 week after last iv PIF)
abnormal (>0.2 μM) in 20% (even several weeks after iv PIF)
PIF: 2-6% labile iron (mostly chelatable within < 1hr by adding apotransferrin).

Conclusions Parenteral iron formulations contain a small but significant fraction of redox-active iron, most of which is scavenged by apo-Tf within <1 h. Therefore, oxidant stress associated with iron infusion is likely to be transient. The bulk of the polymeric iron is apparently inaccessible to apo-Tf. Although LPI might return to normal within 2 h of intravenous iron infusion, the long-term persistence of low-level LPI in up to 20% of end stage renal disease (ESRD) patients indicates that complete clearance of the intravenous iron may be more protracted than originally estimated.

Nephrol Dial Transplant (2004) 19: 561–565
DOI: 10.1093/ndt/gfg579

Labile iron in parenteral iron formulations: a quantitative and comparative study

David Van Wyck[1], Jaime Anderson[2] and Kevin Johnson[2]

Approximately 2–6% of total iron in commonly used IV iron compounds is biologically available or labile iron for in vitro iron donation to Tf. This fraction may contribute to evidence of bioactive iron in patients after IV iron administration.

A recent unreviewed report, using sophisticated fluorescent methods to measure *in vitro* Tf-iron binding, showed that uptake of iron by apotransferrin from IV iron agents is rapid in the presence of ascorbate [4].

Corroborates earlier findings that 2-6% of iron in PIFs is labile and that within an hr of exposure to apotransferrin Tf (in vitro or vivo) it is rendered non-labile (by binding to Tf)

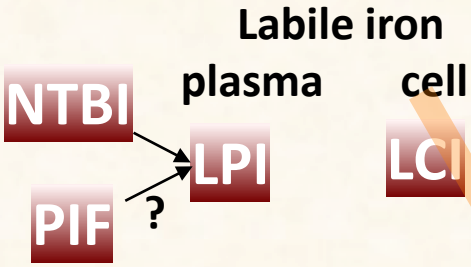
PIF administration
"considerations"

To be determined :

a. % contamination of new PIF with labile iron, effect of storage, $t_{1/2}$ in circulation as LPI.
b. the potential sensitivity of patients with high levels of plasma oxidants (e.g. chronic diabetes) to PIFs .
c. potential risks of PIF retained in the RES (liver and spleen) for extended periods of time and its possible spillover to pancreas in ~ 10% of ESRD patients with high ferritin levels.

Conclusion

In systemic hemosiderosis (hereditary/transfusional/iatrogenic- iv PIF supplements), potentially toxic forms are detected as labile plasma iron (LPI, which is comprised predominantly of Fe(III)). Labile iron can infiltrate cells from plasma via "un-regulated"-resident divalent cation channels/transporters or via endocytosis (if associated with macromolecules). Excessive ingress of labile iron raises LCI and promotes ROS formation .



Diverse ingress mechanisms of plasma NTBI/LPI into cells

Transporter	substrate	auxiliary
•Zip14	Fe(II)	Fe-reductase
•L-Ca channels	Fe(II)	Fe-reductase
•endocytosis	Fe➢protein PIFS*	

*Sohn Y-S et al. he role of endocytic pathways in cellular uptake of plasma NTBI . Haematologica 2012;97(4):670-678.