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

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Disclosure of Interests

- Consultant for Aferrix and Hinoman, Tel Aviv, Israel
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**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\(_2\)O\(_2\) and O\(_2\)^-\), 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, glutathione and carboxylates (di-OH benzoates?).
- LCI levels are determined by the redox potential (NADPH, NADPH, GSH) of the respective cell compartments
- LCI cytosolic (LCI\(_c\)) 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 \([\text{Fe(II)} \rightleftharpoons \text{Fe(III)}]\)
- 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)\(\rightleftharpoons\)Fe(III) conversion.
LABILE CELL IRON (LCI)

overload, toxicity and cell death types

LPI → ↑ LCI levels + ↑ ROI → ↑ ROS → oxidative damage

Fe(II) ⇌ Fe(III) + O₂⁻ + H₂O₂ → O₂ + OH⁻ + OH⁻

ferroptosis oxytosis necrosis cell death

↑ chelators

anti-oxidants

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

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

↑ protecting agents

MI= maldistributed iron

MI= maldistributed iron

KDIGO
**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.

\[ \text{SIO} \uparrow \text{LPI} \rightarrow \uparrow \text{LCI} \rightarrow \text{ROS} \uparrow = \text{oxidative damage} \]

- **ferroptosis**
- **oxytosis**
- **apoptosis**
- **necrosis**
- **chelators**
- **antioxidants**
- **anticaspases**
LABILE IRON IN BIOLOGICAL SYSTEMS
SELECTED REFERENCES (from Cabantchik, Z.I et al.) and their links


*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**
  - DHR
- **H$_2$O$_2$**
- **DHR oxidizes to fluorescent R by ROS generated from LCI prompted with H$_2$O$_2$**

**The recovery of CALG fluorescence $\Delta F$ elicited by a chelator reveals CAL-Fe, which is $\approx$ LCI**
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

transferrin

NTBI, mostly protein adsorbed

40 µM

2 µM

1. Extraction via “mild” chelation (80 mM NTA)

2. filtration

accurate, sensitive, reproducible.

3. Detection with sensor (± HPLC)

Hershko el 1978; Hider, Porter et al 01

extraction & filtration

laborous; 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’.

LPI: labile plasma iron
Esposito et al 03

that binds to bleomycin
that oxidize DNA or deoxyribose
and promotes generation of oxidative radicals

Fe²⁺

bleo

no Fe chelator → yes blocks

ascorbate reduces LPI to Fe(II)

DHR

non-fluorescent substrate DHR to fluorescent product R

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

High throughput fluorescence assay for any LI-containing fluid

color product
detection with TBA
Thiobarbituric acid

40 µM

2 µM

TBI

NTBI

Problematic!
Estimates of LPI and NTBI in patients with "iron overload"

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

<table>
<thead>
<tr>
<th>Patient group</th>
<th>LPI</th>
<th>NTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalassaemia (intermed. 30%, major 50%)</td>
<td>0.5–10</td>
<td>0.5 to &gt;10</td>
</tr>
<tr>
<td>Hereditary haemochromatosis 15% (6-10 weeks after last phlebotomy)</td>
<td>0.2–2.0</td>
<td>0.1–5.0</td>
</tr>
<tr>
<td>MDS (transfused) ~ 30%</td>
<td>0.5–2.5</td>
<td>0.1–3</td>
</tr>
<tr>
<td>• Chronic (advanced) diabetes ~ 35%</td>
<td>0.2–2.2</td>
<td></td>
</tr>
<tr>
<td>• Random controls &lt; 1%</td>
<td>0.2–0.6</td>
<td>-0.2–1.0</td>
</tr>
</tbody>
</table>

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.
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
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


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.
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.

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).
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.
**Conclusions**

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.