LPI-labile plasma iron in iron overload

Z. Ioav Cabantchik* MD, PhD
Professor of Biochemistry and Head of Life Sciences Program

William Breuer PhD
Research Associate
Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, 91904 Israel

G. Zanninelli MD
Attending Physician

P. Cianciulli MD
Director
Unita’ Day Hospital Talassemici, Ospedale Sant’ Eugenio, Rome, Italy

Labile plasma iron (LPI) represents a component of non-transferrin-bound iron (NTBI) that is both redox-active and chelatable, capable of permeating into organs and inducing tissue iron overload. It appears in various types of hemosiderosis (transfusional and non-transfusional) and in other iron-overload conditions. Sustained levels of LPI could over time compromise organ (e.g. heart) function and patient survival. With the advent of methods for measuring LPI in the clinical setting, it has become possible to assess the implications of LPI in the management of iron overload based on regimens of iron chelation. As LPI is detected primarily in patients with transfusional iron overload and other forms of hemosiderosis, we review here regimens of iron chelation with deferrioxamine and deferiprone (separately or combined) in terms of their efficacy in minimizing daily exposure to LPI in thalassemia major and thalassemia intermedia patients.

Key words: labile plasma iron; iron overload; thalassemia; hemosiderosis; transfusion; hemochromatosis; ascorbate; oxidative stress; fluorescence; high throughput assay.

Chronic diseases of iron overload have two distinct features: (1) plasma iron levels become excessively high, and (2) sustained excess of plasma iron leads to iron accumulation in the liver, endocrine organs and heart. Excessive iron accumulation in the respective organ is the cause of liver fibrosis and ensuing hepatic dysfunctions (including hepatocarcinomas), diabetes and cardiomyopathies.1,2

* Corresponding author. Tel.: +972 2 658 5420; Fax: +972 2 658 6974. E-mail address: ioav@cc.huji.ac.il (Z.I. Cabantchik).
The major therapeutic approaches in the treatment of transfusional iron overload or hemosiderosis are based on iron chelators that are assessed in terms of their capacity to reduce body iron burden, primarily by complexing and eliminating liver iron. The clinical efficacy of chelation therapy has been generally evaluated by following up classical parameters such as plasma ferritin (often, but not uniquely, a measure of iron stores) and serum iron (usually in conjunction with total iron binding capacity and/or transferrin saturation). More specific measures of iron overload of clinical relevance have relied on the biochemical and histochemical analysis of biopsies taken from particular organs, usually the liver. Magnetic susceptometry (SQUID) and recently also magnetic resonance imaging (MRI) have offered the possibility of in situ assessment of iron accumulation in liver or heart by non-invasive means. However, hitherto little attention has been given to the nature and source of iron that circulates in the plasma and interstitial fluids and is assumed to be the primary vehicle of tissue iron overload and ensuing toxicity. That source of iron is not identified either with transferrin (which is highly iron-saturated in virtually all the iron-overloaded patients in question) or with ferritin, whose plasma levels generally reflect iron overload per se (e.g. chronic inflammation).

The circulating forms of iron that lead to tissue iron overload and are not tightly associated with plasma transferrin have been generically termed non-transferrin-bound iron (NTBI). In general, NTBI emerges whenever the capacity of transferrin to incorporate incoming iron (from the gut or reticuloendothelial cells) becomes a limiting factor, either because of insufficient binding capacity per se or for kinetic reasons. Although NTBI and labile plasma iron (LPI) appear primarily in highly transfused patients whose transferrin iron-binding capacity has been surpassed, they are often found at transferrin saturations well below 100%. Failure of iron to be incorporated into transferrin results in its association with other plasma components. The chemical structures comprising NTBI might vary with the degree of iron overload, encompassing forms that are readily chelated by commonly used chelating agents as well as others that can be chelated only if they are first extracted from their adsorption sites with high concentrations of mobilizing agents. However, the pathologically relevant fraction of NTBI is that which is seemingly translocated across cell membranes in a non-regulated manner and leads to excessive iron accumulation in liver, heart, pancreas and other endocrine organs. That fraction is referred to as labile plasma iron (LPI) since it encompasses cell-penetrating forms of iron that are redox active and susceptible to chelation. These attributes render LPI not merely an accessible diagnostic marker of iron overload and cell toxicity but also a clinical parameter that could be used for assessing the mode and efficacy of iron chelation.

The design of iron chelators has classically focused on agents aimed at reducing the body iron burden by acting on the major sites of iron accumulation and thus indirectly reducing the levels of the metal in plasma and interstitial fluid. Agents conforming to that design are desferrioxamine (DFO) and ICL670, both of which act directly on liver iron and excrete it via the biliary route, and deferiprone (used either alone or in combination with DFO), which also seemingly extracts iron from liver, but carries it into plasma for secretion via the urinary tract. However, the other mode by which chelators such as DFO and deferiprone might reduce LPI is by direct capture of LPI itself, thus preventing iron entry into organs (such as the heart) of iron-overloaded patients. For such a strategy there might be a need for maintaining constant basal levels
of plasma chelating activity during most hours of the day. A third mode of chelator action relies on the ability of the chelator to permeate extrahepatic tissue such as heart or pancreas and thereby neutralize the cytotoxic activity associated with accumulated iron. Deferiprone and ICL670 apparently fit that category, although the capacity of the chelates to exit cells probably varies considerably between the two agents. Optimal results are expected to be obtained with agents or regimens that display the three modes of chelation, whether based on these same drugs or their combination.

**WHY ANOTHER ASSAY FOR ASSESSING IRON OVERLOAD?**

Despite the success of daily DFO treatment in maintaining thalassemia major patients in negative iron balance over more than three decades, a significant number of patients have apparently developed serious and often fatal heart conditions. In many cases these conditions could be attributed to iron accumulation in the heart rather than the liver due to their low correlations with liver iron or circulating ferritin. The proposed view is that the treatment (in most cases night-time infusion of DFO) might not have precluded repetitive exposure of the heart to circulating forms of labile iron that could lead to critical accumulation of the metal in cardiomyocytes via the divalent metal transporter DMT1 and/or dihydropyridine-sensitive L-type calcium channels. Since this phenomenon seems to be less prevalent or non-existent in patients treated with DFO continuously (24 hours), it is reasonable to assume that any chelation treatment that could attenuate formation of LPI would minimize or prevent the deleterious effects of iron on extrahepatic tissue. This raises the outstanding questions of whether LPI monitoring could be used for assessing the efficacy of iron chelation treatment, and whether any clinical endpoints correlate with measurable LPI levels.

As stated above, LPI refers to that component of plasma NTBI that is redox active, chelatable, and capable of entering (substantially) into cells. This LPI component of NTBI appears primarily in patients with hemosiderosis whose transferrin saturation levels exceed 80\%\, irrespective of the etiological reason. Recent epidemiological studies indicated that cardiac complications related to iron overload are most prevalent in patients with a transferrin saturation higher than 85\%. Such correlations provide the basis for the link between cardiac complications in iron overload and LPI. What they imply for the medical practitioner is that assessment of iron chelation regimes based solely on serum ferritin levels or other classical parameters of liver iron provide only a fragmentary and often misleading picture of the iron status of patients and the quality of treatment. Therefore, despite an apparently adequate liver iron extraction quotient, a daily chelation treatment might still leave wide windows of exposure to LPI that over time could compromise the patient’s health. Can measurements of LPI provide a direct index for the efficacy of a chelation treatment of iron-overloaded patients, and if so, can LPI levels be correlated with the risk of iron accumulation in the heart?

**LPI LEVELS IN THALASSEMIA**

A new high-throughput technology (FeROS, Aferrix Ltd) was recently devised for assessing LPI in the clinical setting and has already been applied to more than 5000 serum samples, including patients with thalassemia (major and intermedia).
hemochromatosis\textsuperscript{22,33}, diabetes\textsuperscript{35}, dysmetabolic hepatosiderosis\textsuperscript{33}, and myelodysplastic syndrome.\textsuperscript{36} The assay is based on the capacity of LPI to generate reactive oxygen radicals when prompted with ascorbate in a manner blockable by specific iron chelators.\textsuperscript{22} LPI was detected in more than 75\% of randomly selected β-thalassemia patients undergoing regular blood transfusions and chelation treatment and non-transfused hemoglobin E (HbE)/β-thalassemia patients. The LPI levels vary from 0.4 to 10 μM, depending on the nature of the disease, the transfusion schedule, the chelation regimen, and the time of blood sampling relative to the drug administration. The following sections provide three examples of LPI measurements in clinical studies aimed at evaluating different regimens of chelation in iron-overloaded patients.

Example 1: LPI in HbE/β-thalassemia patients during an 80-week course of deferiprone treatment (modified from reference 23)

A group of 17 Thai patients (non-transfused and non-chelated for more than 1 year) were subjected prospectively to a course of long-term deferiprone treatment (50 mg/kg/d in two doses, morning and evening) (Figure 1). Bimonthly values of LPI, serum ferritin and red-cell-membrane-associated iron for the entire cohort are depicted as stacked areas. The height of the stack represents the mean value at the indicated time point. Daily administration of deferiprone for 13–17 months caused LPI

![Figure 1. The figure depicts the mean basal LPI, serum ferritin (SF) and red-blood-cell-membrane-associated iron (RBCM-I) values relative to those obtained on entry into the study in 17 HbE/β-thalassemia patients treated with deferiprone orally (50 mg/kg/d in two doses) for up to 18 months; blood samples were withdrawn every 2 months in the morning after at least 10 hour washout of deferiprone. The height of a given area at a particular month depicts the level of the parameter relative to that at the onset of the treatment. The actual mean values of each parameter at the beginning of treatment (month 1) were: for LPI 4.7 ± 0.5 μM; for RBCM iron 8.0 ± 1.5 nmol/mg protein, and for SF 3407 ± 868 ng/ml. Data modified from Pootrakul P et al (2004, Blood in press) with permission.](image-url)
to decrease from an initial corrected mean level of 4.7 ± 0.5 μM (measured before the morning intake of deferiprone) to steady mean levels of 1.8 ± 0.2 μM, attaining steady lowest levels after 6–8 months with a t\(_{1/2}\) of 2–3 months. Serum ferritin and red-cell-membrane-associated iron followed a similar course, but attained steady basal levels only after 10–12 months of continuous treatment with a t\(_{1/2}\) of 5–7 months. On the other hand the percentage transferrin saturation and total serum iron remained elevated throughout the entire course of treatment. This study indicated that persistent levels of LPI values detected during daily drug washout periods can serve as early indicators of iron overload and as a measure of the effectiveness of iron chelation in reducing potentially toxic iron in the plasma of thalassemia intermedia patients. Moreover, the study also showed that the particular regimen of deferiprone applied for more than a year to the indicated set of patients was not sufficient to bring down LPI basal levels to the threshold level of 0.6 μM which represented the 0.4 μM ± 0.2 SD detection sensitivity level of the assay. A possible explanation is that, for the group of patients in question, the rate of LPI removal by a 50 mg/kg/d dose of deferiprone was in steady state with the rate of iron replenishment. Further reduction in LPI might demand an intensified treatment based on higher deferiprone dosage, possibly more spread out during the day. However, it should be stressed that the clinically relevant threshold levels of LPI, whether assessed as basal levels or in terms of cumulative daily exposure to LPI, still need to be more strictly defined in carefully planned longitudinal studies.

**Example 2: daily LPI levels in β-thalassemia patients undergoing different iron chelation treatments**

A group of 30 thalassemia major patients, who had been treated for years with DFO only (40 mg/kg/d subcutaneously), were subdivided into three treatment groups (n = 10 each): one continued with DFO overnight, one switched to daily deferiprone taken orally (75 mg/kg/d in three doses), and one switched to a combination of daily deferiprone and overnight DFO (Figure 2). Patients were chosen for treatment with deferiprone because of compliance problems with DFO, and for treatment with DFO plus deferiprone because of persistently high serum ferritin levels despite the previous DFO treatment. The individual daily profiles of LPI for each group obtained after more than 12 months of treatment are shown in Figure 2 (samples were taken every 2 hours during the day, from 8 a.m. until midnight, and next day at 8 a.m.—indicated as 32 hours, and where applicable—before drug intake). The individual profiles are stacked one above the other, and the height of each stack represents the actual LPI value at the indicated hour of treatment.

**For DFO-treated patients**

LPI was essentially undetectable during the course of DFO infusion (Figure 2, top), while it gradually rose afterwards, peaking at evening hours just before the next infusion. In seven out of ten patients the LPI levels did not rise above 0.6 μM during the day, in two they rose moderately during the late afternoon and early evening hours, and in one they rose considerably within 2 hours after treatment and thereafter throughout the day until the next infusion.

**For deferiprone-treated patients (Figure 2, middle)**

The mean basal LPI levels (detected after a 10-hour washout period, namely, after the last deferiprone dose) were in the range 0.6–1.4 μM on two consecutive days. The basal
Figure 2. Daily LPI levels in individual thalassemia major patients treated with different chelators. The following depict the individual LPI values obtained in groups of 10 thalassemia major patients (individually indicated by a different color) treated either with DFO subcutaneously at 40 mg/kg/d (top: blue box—duration of infusion), deferiprone orally (75 mg/kg/d) (middle: separate doses indicated by arrows) or combined therapy (bottom: arrows for deferiprone and blue box for DFO infusion). Blood samples were taken at 2-hour intervals from 8 a.m. to midnight (the 32-hour point represents the sample 24 hours after the first sample was taken). The area under each line represents individual results stacked one above the other. The height of a given area at a particular time depicts the level of LPI at the indicated hour of sampling. The white mesh represents the 0.4 precision limit of the assay or background level found in sera from normal individuals. From Zanninelli et al (2004, Proceedings of the European Iron Club meeting, Rennes 2004) with permission.
LPI values were found to be representative of the individual profiles. In five out of ten patients the basal LPI levels were significantly higher than the threshold value of 0.6 \( \mu \text{M} \); LPI generally fell after deferiprone intake but gradually rose back during washout periods longer than 2 hours. In the remaining five patients LPI levels fluctuated (i.e. fell after drug intake and rose gradually during the washout period), but were maintained within the lower 0.6 \( \mu \text{M} \) range, indicating the adequacy of the standard deferiprone monotherapy for this subgroup of patients.

For deferiprone-plus-DFO-treated patients (Figure 2, bottom)

LPI levels did not exceed the 0.6 \( \mu \text{M} \) threshold level during the entire 24-hour follow up. In fact, in all cases except one the levels were maintained even below the 0.4 \( \mu \text{M} \) range.

The implication of the above results is that the washout levels of LPI provide a good measure of the general efficacy of the chelation treatment. On the basis of the known pharmacokinetics of deferiprone and DFO, a chelator washout period of 4 hours was already sufficiently long to preclude interference with LPI determination. This entailed sampling at about 8 a.m. for deferiprone-treated patients (previous deferiprone taken the night before) and 8–10 p.m. for patients treated with DFO alone or in combination with deferiprone (previous deferiprone dose taken at midday and DFO infusion completed in the early morning). The mean basal (post-washout) LPI values correlated linearly (\( r^2 = 0.90 \)) with the respective mean levels of serum ferritin attained with the different treatments. The analysis yielded a linear correlation with a slope of 0.40 \( \pm 0.12 \) \( \mu \text{M} \) per \( \mu \text{g/ml} \) ferritin (typical pairs of LPI in \( \mu \text{M} \) and ferritin \( \pm 0.06 \mu \text{g/ml} \)) values were: 0.25 \( \pm 0.06 \) and 0.456 \( \pm 0.092 \) for deferiprone-plus-DFO; 0.69 \( \pm 0.22 \) \( \mu \text{M} \) and 1.881 \( \pm 0.665 \) for DFO alone, and 0.95 \( \pm 0.27 \) and 2.006 \( \pm 0.416 \) for deferiprone alone. Our experience with the relatively small cohort of patients engaged in the study (ten per group) indicates that the combination of deferiprone-plus-DFO provided not

![Figure 2 (continued)](https://example.com/figure2 continued)
only the lowest LPI values throughout the entire day, and the lowest serum ferritin values, but gave also the most uniform response to the chelation treatment.

**Example 3: LPI levels in β-thalassemia patients undergoing treatment with the daily dose of deferiprone divided into three versus four**

This study was aimed at assessing the possibility of diminishing the daily LPI levels by spreading out the oral deferiprone intake over the day (same final dose, but divided into four instead of three doses per day) (Figure 3). For that purpose, the same group of thalassemia major patients who were treated previously with 75 mg/kg/d deferiprone in three daily doses (Figure 2) were switched to four doses and their mean daily LPI levels compared (Figure 3). The mean basal levels of LPI (at 8 a.m. and, 24 hours later, at 32 hours) remained essentially the same for both treatments (1.15 ± 0.20 μM; range 0.81–1.41). However, with four daily deferiprone doses the mean LPI level did not exceed the 0.6 μM level during the day (until midnight), whereas with three doses it significantly exceeded that level by evening (18–24 hours). Whether spreading out the total daily dose of deferiprone has a similar effect on independent parameters of body iron status/burden remains to be determined.

**Figure 3.** Mean LPI levels in thalassemia major patients treated with the same total daily dose of deferiprone divided differently. The following depicts the mean LPI ± SEM values obtained from groups of nine or ten thalassemia major (same) patients treated with deferiprone orally (arrows) in three versus four daily doses. The height of a given area at a particular time depicts the level of the parameter at the indicated hour of sampling (every other hour from 8 a.m. until midnight). Time of sampling/treatment represents the time of day (in hours) at which either blood samples were withdrawn or drug was delivered. LPI exceeded the 0.6 μM threshold: (a) 8 (or 32), 18, 20 and 24 hours for the three-dose group, and (b) 8 (or 32) for the four-dose group. The white mesh represents the precision limit of the assay or background level found also in sera from normal individuals. Modified from Zanninelli et al (2004, *Proceedings of the European Iron Club meeting, Rennes 2004*) with permission.
SUMMARY

LPI values obtained following an extended drug washout period (basal LPI levels) provide a means for evaluating the efficacy of treatment in maintaining the individual at low levels of exposure to labile plasma iron.

LPI values vary among iron-overloaded patients in relation to the regimen of chelation and time of blood withdrawal. Although the basal LPI levels decline relatively faster than other iron-related parameters, they correlate linearly with serum ferritin levels as well as with red-cell-membrane-associated iron. The combined deferiprone-plus-DFO treatment yield the lowest basal LPI values and serum ferritin values, as compared to those obtained with DFO or deferiprone alone.

LPI levels were considered highly significant in terms of iron overload if they exceeded by 100% the assay mean ± SD background/noise level of 0.4 ± 0.2 μM, i.e. if LPI levels were > 0.6 μM. Unlike combined deferiprone-plus-DFO-treated patients, who showed no significant LPI during the entire day, those treated with either DFO alone or deferiprone alone showed a non-uniform behavior with respect to daily LPI levels. Some patients subjected to monotherapy were found not to have significant basal LPI levels at any time during the day, whereas others had only after prolonged washout times, and some during most hours of the day.

The individual daily basal values of LPI or daily profiles of LPI can be used for optimizing chelation treatment (dosage regimen: number of doses per day, single or combined therapy, etc.) so as to attain a full-day coverage of LPI-preventing activity. In the deferiprone-treated group a significant improvement in diminishing daily LPI was achieved just by spreading the daily treatment from three to four deferiprone doses. However, the clinical outcome of spreading the deferiprone treatment remains to be further assessed.

Practice points

- for clinical trials or preliminary assessment of deferiprone and/or DFO regimens of treatment, the incorporation of LPI assays should be based on a selected schedule of blood sample withdrawals (followed by serum preparation, separation and deep-freeze storage; ca. 0.5 ml total serum sample)
- the number of blood samples to be taken might depend on the availability of patients. All samples should be taken before drug intake (orally or by infusion) and if possible at different washout periods: (a) at the onset and end of treatment it might be useful to obtain a complete daily profile: 2–10 samples during a 24-hour period (7–8 a.m. to midnight, 2 or 12 hours apart and 24 hours later); if only one or two daily samples are available, then one should consider sampling after a drug washout period of at least 4 hours for deferiprone or for DFO, preferably on two consecutive days; (b) during treatment, one blood sample (basal LPI) every 2 months—or as often as required or available—in the morning before deferiprone intake and before DFO infusion
- analogous schedules can be designed for assessing ICL670 as well as other novel iron chelators
ACKNOWLEDGEMENTS

This work was supported in part by The European Community 5th Framework QLRT-2001-00444 (nutrient iron toxicity) and by Apopharma Inc, Ont., Canada.

REFERENCES


