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Daily labile plasma iron as an indicator of chelator activity in *Thalassaemia major* patients

Giuliana Zanninelli,[1] William Breuer[2] and Zvi I. Cabantchik[2]

[1]Unita' Day Hospital Talassemici, Ospedale Sant' Eugenio, Rome, Italy, and [2]Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel

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Correspondence: Professor Zvi I. Cabantchik, Adelina and Massimo Della-Pergola Chair of Life Sciences, Department of Biological Chemistry, Hebrew University of Jerusalem, Safra Campus – Givat Ram, Jerusalem 91904, Israel. E-mail: ioav@cc.huji.ac.il

Summary

Labile plasma iron (LPI), a non-transferrin-bound component of plasma iron detected in iron overload disorders is a potential source of cellular iron accumulation and ensuing oxidative damage. Periodic monitoring of LPI over a 24 h time-span was used to compare the ability of chelation to control daily LPI levels in 40 Thalassaemia major patients (9–11/group) who had been receiving one of three different chelation protocols for more than a year: Group I. deferrioxamine overnight, Group II. deferiprone daily, Group III. deferrioxamine and deferiprone sequentially. An additional group (Group IV) was treated with desferasirox for up to 6 months. The patterns of daily LPI recrudescence showed significant individual variations, especially in patients treated with deferrioxamine or deferiprone, although these patterns were maintained over 6–9 months of treatment in all groups. Group data analysis showed that the proportion of patients whose daily LPI were maintained within the normal range (<0·45 μmol/l) varied with treatment: 6/10 with deferrioxamine, 5/11 with deferiprone, 9/10 with deferrioxamine + deferiprone and 8/10 at the onset and 10/10 after 6 months treatment with deferasirox. Although the clinical significance and therapeutic value of LPI remain to be established, monitoring of daily LPI level may provide an analytical basis for assessing chelation efficacy in preventing daily LPI recrudescence.

Keywords: iron, chelation, thalassaemia.

Secondary iron overload, which develops in *Thalassaemia major* patients as a result of multiple blood transfusions, eventually leads to uncontrolled iron release into the circulation, ultimately surpassing the plasma transferrin iron-binding capacity and generating a variety of complexes collectively labelled as non-transferrin bound iron (NTBI) (Hershko *et al*, 1978; Hider, 2002; Breuer *et al*, 2000). A fraction of NTBI that is redox active and chelatable is referred to as labile plasma iron (LPI) (Breuer *et al*, 2000; Cabantchik *et al*, 2005; Esposito *et al*, 2003; Pootrakul *et al*, 2004). Some forms of NTBI, such as the LPI fraction, can also permeate into cells opportunistically via various trans-membrane pathways, thereby raising the labile cell iron above steady state levels and catalysing the generation of reactive oxygen species (ROS) (Glickstein *et al*, 2006). Persistent exposure to ROS may eventually override the cell antioxidant capacity and lead to tissue oxidative damage that results in vital organ dysfunction.

The above scenario of systemic iron overload can be therapeutically addressed by targeting: a. the sources of iron overload, by chelating the LPI in the plasma; b. the vehicles of iron overload, by blocking LPI's membrane permeation paths and c. the consequences of iron overload, by chelating and eliminating labile cell iron and thereby the tissue-accumulated metal. Previous studies indicated that high affinity iron chelators in clinical use act via mechanisms (a) or (c) or both and that their efficacy depends largely on their pharmacokinetic and membrane permeation properties (Porter, 2005; Cappellini & Pattoneri, 2009; Galanello, 2005), particularly their ability to access labile iron in critical cells of the heart and endocrine glands and bring about a reduction in the accumulated iron (Glickstein *et al*, 2005, 2006).

A major premise of this work is that LPI control is an essential component of preventive-chelation therapy, as LPI can be considered the major source of iron overload and

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ensuing peroxidative damage of vital organs. This was first demonstrated in studies aimed at assessing the short-term and long-term efficacy of deferiprone (DFP) treatment in *Thalassaemia intermedia* patients and corresponding changes in serum ferritin levels and other blood parameters (Pootrakul *et al*, 2003, 2004). A preliminary study indicated that whereas DFP or deferrioxamine (DFO) treatment gave *Thalassaemia major* patients only partial daily protection from LPI emergence, their combination was considerably more efficacious (Cabantchik *et al*, 2005). In previous (Zanninelli *et al*, 2007) and more recent reports (Daar *et al*, 2009; Porter *et al*, 2008; List *et al*, 2007), the long term effects of deferasirox (DFR) on groups of patients with either *Thalassaemia major*, myelodysplastic syndromes or rare anaemias, were presented, mostly as changes in the group mean values of LPI ± SD.

It is our view that whereas mean LPI ± SD levels in a cohort of patients might be useful for depicting the chelation activity attained in plasma and/or the statistical significance of a particular treatment, they do not necessarily provide a picture of the adequacy of treatment in individuals or the singularity of a patient's response. In fact, responses to treatment are expected to vary among patients relative to the pharmacokinetics of the chelator and the patient's iron status. In this study we performed periodic daily monitoring of LPI to assess the extent to which established chelation protocols (Porter, 2005; Cappellini & Pattoneri, 2009; Galanello, 2005) eliminate LPI recrudescence in individual β-*thalassaemia major* patients. The abrogation of LPI could provide a useful early indicator of the effects of a chelator regimen, while establishing a foundation for customizing and optimizing chelation therapy.

Methods

Reagents

Reagents for LPI assays were: ascorbic acid, ferrous ammonium sulphate, bovine serum albumin Fraction V (Sigma, St Louis, MO, USA), dihydrorhodamine 123, dihydrochloride salt (DHR) (Biotium, Hayward, CA, USA), DFP (Deferiprone; Apotex, Mississauga, ON, Canada), nitrilotriacetic acid (NTA) (Fluka, Seelze, Germany), deferrioxamine (DFO; Novartis, Basel, Switzerland) HEPES-buffered saline (HBS; HEPES 20 mmol/l, NaCl 150 mmol/l, pH 7·4). Ascorbate (free acid form) was prepared as a concentrated stock of 20 mmol/l in water. DHR was prepared as a concentrated 50 mmol/l stock in acidified dimethyl sulphoxide (produced by adding 0·05 ml of 2 mol/l HCl to 1·0 ml of dimethyl sulphoxide).

Analyses

Chemical measurements of liver iron (Torrance & Bothwell, 1968) and serum ferritin (Flowers *et al*, 1986) were carried out as described in these studies and serum iron and total iron binding capacity were measured as recommended by the International Committee for Standardization in Hematology (ICSH, 1978).

LPI assay

The method is based on the conversion of the non-fluorescent dihydrorhodamine (DHR) to the fluorescent form by various oxidants, such that the generation of reactive oxygen species can be followed as an increase in DHR fluorescence (Esposito *et al*, 2003). In the LPI assay, each serum sample was tested under two conditions: with 40 μmol/l ascorbate alone and with 40 μmol/l ascorbate in the presence of 50 μmol/l chelator, such as DFP or DFO. The difference in the rate of oxidation of DHR in the presence and absence of chelator represented the component of plasma NTBI that is redox-active and chelatable. The slopes (r) of DHR fluorescence intensity with time were calculated from measurements taken between 15 and 40 min.

LPI calculation. Duplicate values of r in the presence and absence of DFP, r_{DFP} and r respectively, were averaged and the LPI concentration (μmol/l) was determined from calibration

Table I. Entrance parameters and summary of patients enrolled in the study.

Treatment group	Sex	Age (years)	Ferritin (μg/l)	Iron (μmol/l)	TF SAT (%)	ALT (Units)	HCV RNA+	LIC (mg Fe/g)	Cardiac
I. DFO	8F/2M	36 ± 7	1881 ± 2020	30 ± 7	68 ± 14	68 ± 72	5	4·5 ± 3·5	2A
II. DFP	5F/5M	32 ± 7	1696 ± 955	32 ± 9	77 ± 20	34 ± 23	6	3·7 ± 2·1	2A, 1C
III. DFO + DFP	5F/5M	36 ± 9	456 ± 294	30 ± 9	70 ± 26	69 ± 80	5	5·1 ± 3·1	7A, 4C
IV. DFR	7F/3M	32 ± 10	2869 ± 2620	50 ± 29	95 ± 27	83 ± 67	5	9·1 ± 4·4	1A, 2C

The mean values (±SD) of most relevant parameters of the patients selected for the four groups of treatment (10 patients per group) are indicated. The various entrance parameters and indicators were obtained as described in *Methods*: TF SAT, transferin saturation; ALT, Alanine transaminase; HCV, hepatitis C virus; LIC, liver iron concentration (normal range <5 mg Fe/g dry tissue). Patients from Groups I, II and III had been on the same treatment for more than 1 year prior to entering the study, whereas those from Group IV have been previously treated with either DFO or DFP. The number of patients with a cardiac condition [cardiopathy (C) and/or arrhythmia (A)] is indicated for each treatment group. The cardiac assessment comprised standard echocardiographic (ECG) parameters, ECG and ECG 24 h (Holter), ejection fraction and symptomatic heart failure. Cardiopathy was indicated when atrial and ventricular diameters were out of normal range. Arrhythmias mostly consisted of atrial fibrillation and ventricular extrasystolic beat.

curves indicating the difference in slopes with and without DFP against Fe concentration (using a Fe:nitrilotriacetate complex with 1:10 stoichiometry). Normal sera typically yield LPI values of 0·2 ± 0·2 μmol/l, however in some cases, such as haemolytic sera, the LPI values may rise to 0·25 ± 0·2 μmol/l, causing the apparently normal range of LPI levels to increase up to 0·45 μmol/l (Esposito *et al*, 2003; Pootrakul *et al*, 2004). On this basis, all LPI values ≤0·45 μmol/l were considered to be within the normal range, while values >0·45 μmol/l were considered LPI-positive. Some of the LPI assays were carried out with a recently commercialized kit (Aferrix, Tel Aviv, Israel) based on Esposito *et al* (2003).

Patient treatment and blood sampling

Forty transfusion-dependent β-thalassaemia patients from the Thalassaemia Centre of St Eugenio Hospital in Rome, Italy, treated with DFP, DFO, DFP and DFO or DFR were enrolled in the study (Table I). The patients were divided into the following four groups according to their therapy: *Group I*, patients receiving DFO (40 mg/kg per day) subcutaneously overnight from 8:00 PM until 6:00 AM ($n = 10$); *Group II*, patients receiving DFP (deferiprone, 75 mg/kg per day) in three daily doses (as indicated), after meals ($n = 10$); *Group III*, patients receiving DFP during the day and DFO during the night at the same dosage as Groups 1 and 2 ($n = 10$); *Group IV*, patients receiving DFR (30 mg/kg per day) once daily in the morning *per os* before meals ($n = 10$). The patients in *Groups I* and *III* and those who entered *group IV* after being on DFO, were instructed to stop the infusion pump with DFO before 6:00 AM on the day of the blood test. All the patients were instructed to arrive to the hospital at 7:30 AM on the day of the study and their blood was collected every 2 h from 8:00 AM (before the intake of DFP or DFR until 8:00 PM, and the next morning at 8:00 AM. All patients received their last blood transfusion at least 2 weeks prior to being admitted to the present study in order to exclude the presence of exogenous LPI. The patients enrolled in the study underwent a complete checkup for serum iron, serum transferrin, serum ferritin, alanine transminase (ALT) and LPI. Written informed consent was obtained from each patient in accordance with procedures established by the Sant'Eugenio Hospital, and the study was approved by the hospital Institutional Review Board. Because of local ethical restrictions, only selected patients with apparent difficulties in handling a particular treatment with either DFP or DFO were allowed to be transferred to the DFR group (IV) whereas all others in Groups I–III (DFO, DFP or their combination) continued the same treatment as it was considered adequate on the basis of other criteria.

Daytime versus night-time LPI

In order to calculate mean LPI values, the data were divided into day- and night-time LPI values. 'Day-time' values included measurements during the period 2–12 h (10 AM–8 PM), while 'night-time' values referred to a single measurement obtained at the 24-h time point (8 AM the following morning). Therefore, the night-time value represents the accumulation of LPI overnight rather than a calculated average of nightly LPI fluctuations.

Statistical analysis

Statistical and regression analyses were performed using the programme ORIGIN (v. 8.0) OriginLab, Northampton, MA, USA. Statistical parameters were analysed using the two-sample t test, using a P value of 0·05 as the threshold for statistical significance.

Results

The primary goal of this study was to assess the adequacy of the various clinical protocols in maintaining LPI levels of thalassaemia patients within the previously established normal range (<0·45 μmol/l) (Cabantchik *et al*, 2005; Esposito *et al*, 2003; Pootrakul *et al*, 2004). The selected cohort of 40 *Thalassaemia major* patients had already been undergoing standard chelation therapy protocol for more than a year and continued the same treatment during the 24-h LPI-monitoring study: DFO nocturnally (Group I), oral DFP in multiple daily doses (Group II) or combination therapy with DFO and DFP consisting of protocols (I) and (II) (Group III). The DFR group (Group IV) comprised of patients that had been previously treated with either DFO or DFP and transferred to DFR at entrance to this study, following a chelator washout period (of 2–3 h after DFO and 10 h after DFP).

The clinical characteristics of the *Thalassaemia major* patients enrolled in the study are described in Table I. The Table reports the sex, age and clinical parameters at entrance, including serum ferritin, serum iron, transferrin saturation, transaminases, hepatitis C virus (HCV) status, liver iron concentration and the presence or absence of cardiac complications.

A global view of the daily mean LPI profile in the four groups of patients undergoing DFO, DFP, combined therapy or DFR is presented in Fig 1. For Groups I–III, the daily LPI profiles are regarded as steady state, in as much as the patients were more than 12 months on the same treatment and because similar daily profiles were essentially recapitulated 6–9 months later. In *Group I*, receiving overnight infusion with DFO (completed about 2 h before the 0 h LPI sample at 8 AM). LPI was undetectable at 0 h, but afterward rose steadily during the day until the next DFO infusion. In *Group II*, receiving DFP, two major rises in LPI were observed, one in the evening and one in the morning, both after relatively long 'washout' periods (between drug intakes). As expected, following each DFP intake, LPI levels decreased but thereafter rose gradually over the next 2–3 h. Spreading the same daily DFP dose (75 mg/kg) from three to four doses every 4 h offered more effective control of the LPI rebound between

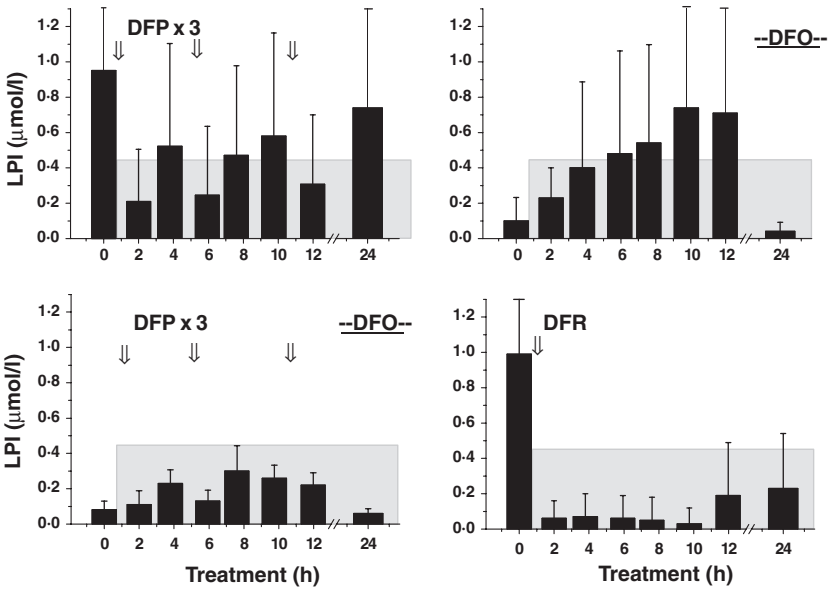

Fig 1. Time-dependent changes in plasma LPI levels of *Thalassaemia major* patients treated with different chelators over a 24-h period. Forty *Thalassaemia major* patients were divided into four groups of chelation regimens (n = 9–11 patients per group) and monitored for LPI levels over a 24-h period. LPI was determined in blood samples taken every 2 h, starting at 8 AM (0 h = entrance value) until 8–10 PM and ending at 8 AM next day (24 h). The chelation regimens were: DFO (40 mg/kg per day given s.c. during the night starting after 9–10 PM) (Group I); DFP (75 mg/kg per day) given orally in three daily doses (times indicated by arrow) (Group II); DFP in three daily doses followed by DFO during the night (Group III); DFR (30 mg/kg per day) given orally once daily (Group IV). Mean LPI values of each group at each time point are given as μmol/l ± SD. The grey-shaded area denotes the normal range of LPI as defined in the *Methods*.

daily doses, causing the mean daily LPI value (μmol/l) to drop significantly, from 0·66 ± 0·14 to 0·50 ± 0·12, and the daily accumulated LPI (μmol/l) from 5·33 to 3·99 ($P < 0·003$) (4). In Groups III and IV, the combined DFP/DFO therapy (Group III) and single daily dose DFR (Group IV) were effective in maintaining mean LPI levels below the 0·45 μmol/l threshold throughout the entire day.

Although the data shown in Fig 1 provide an overall picture of drug efficacy in a group of patients, they do not necessarily depict the adequacy of a given treatment for maintaining daily and/or overnight LPI levels within the normal range in individuals. For this we conducted a statistical analysis using the 40 individual patients' 24 h LPI profiles (individual profiles of 36 of the patients are shown as Figs S1–S4). The LPI daily load was analysed as mean daily levels and overnight levels (see *Methods*) because they provide a measure for the two half-day LPI average levels to which tissues might be exposed. The distribution profiles of mean daily and overnight LPI values among patients are shown as histograms in Fig 2. They indicate that the relative efficacies of the treatments in suppressing LPI recrudescence over 24 h were in the order DFP/DFO (combined) > DFR > DFO > DFP. However, verification of this finding remains to be established by studies based on larger cohort of patients matched for their baseline characteristics and selected to undergo a particular chelation treatment in a non-randomized fashion.

In DFP-treated patients, the major LPI burden occurred after overnight washout (6/10 cases), while during the day mean LPI rose above the normal range in only 2/10 cases. On the other hand, in DFO-treated patients, the major LPI burden occurred during the day, with mean LPI rising above the normal range in 4/10 cases, while overnight LPI values were kept low. In the DFR-treated group, 2/10 patients showed elevated LPI after overnight washout at the onset of treatment, however, after 6 months of continuous DFR treatment their LPI appeared to have been effectively suppressed. In the DFP/DFO (combi) group, only a single patient showed elevated mean LPI during the day. It is noteworthy that of the 40 patients engaged in this study, >80% of those suffering from the various heart complications indicated in Table I had mean daily or mean nightly LPI values >0·5 μmol/l. Finally, in the whole patient cohort we found no statistically significant correlation ($r > 0·5$) between the mean daily or nightly levels of LPI and either the per cent transferrin saturation or the serum ferritin levels. However, we noticed that in at least 90% of patients with mean daily or nightly LPI > 0·5 μmol/l, the transferrin saturation was >75% and the serum ferritin levels were >1000 μg/l.

Discussion

In *Thalassaemia major*, as in other iron-overload diseases, the circulating levels of serum iron are continually replenished by the enhanced iron efflux from iron-storage organs, such as the liver and spleen and by iron hyper-absorption from the gastrointestinal tract (Porter, 2005). LPI, the plasma iron

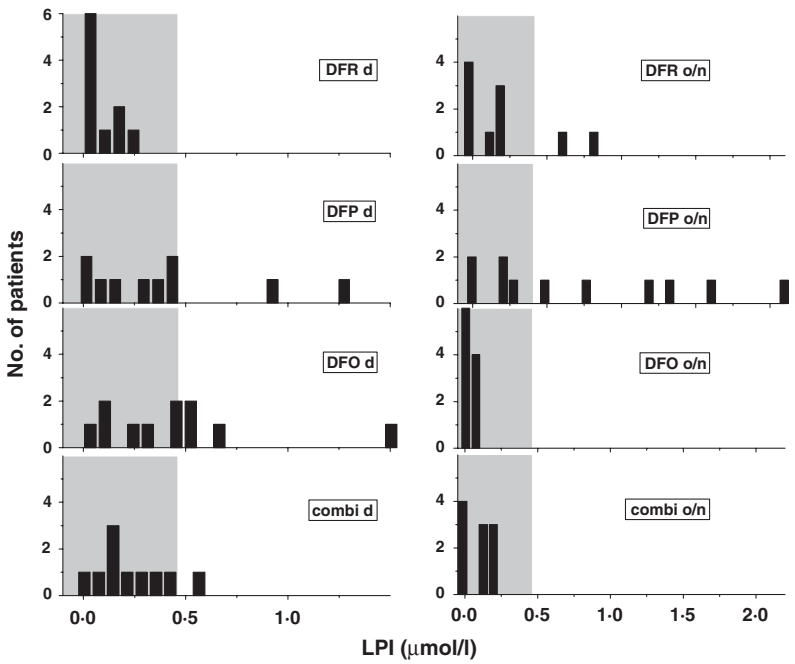

Fig 2. Distribution of mean LPI levels at daytime (d = 8:00–20:00 h) and at overnight (o/n = 22:00–8:00 h) within patient groups treated with different chelators. LPI was monitored in Thalassaemia *major* patients as described in previous figures. Mean daily LPI values were calculated from samples taken at 2–12 h (indicated as d) and overnight LPI values (indicated as o/n) were obtained from the 24 h sample. The graph depicts the distribution of LPI levels among the 10 patients in each group treated with the indicated regimen of chelation (Group I–IV, 10 patients per group). Each bar represents the number of patients for which a particular LPI value was measured. The grey-shaded area denotes the range of LPI values obtained in an apparently normal population. Mean daytime LPI exceeded the normal range (<0·45 μmol/l) in 4/10 patients treated with DFO; 2/10 treated with DFP; 0/10 treated with DFP + DFO and 0/10 treated with DFR. Night-time LPI exceeded the normal range levels in: 0/10 patients treated with DFO; 6/11 patients treated with DFP; 2/10 treated with DFP + DFO and 2/10 treated with DFR.

component whose redox activity is inhibited by exogenously added chelators, may be regarded the earliest measurable parameter affected by chelator ingress into body fluids. This is in contrast to the generally used indices of body iron stores, such as serum ferritin or transferrin saturation, which respond to chelator treatment over periods of weeks to months (Kattamis *et al*, 2006; Mourad *et al*, 2003; Alymara *et al*, 2004). Thus, monitoring daily LPI levels offers several advantages: (i) rapid assessment of a chelation protocol's ability to control this potentially toxic iron fraction, (ii) indexing the duration of chelator action, via detection of LPI re-emergence in the course of chelation treatment and (iii) the possibility for streamlining the customization of chelation protocols.

In this work we assessed the comparative efficacy of four established chelation treatments in terms of their ability to abrogate daily LPI resurgence in individual *Thalassaemia major* patients. The 40 participants entered the study because they had already been on a given treatment for more than a year, 30/40 continued the same treatment and 10/40 were switched to treatment with DFR. A follow-up of daily LPI levels enabled the determination, with minimal delay, of the attainment of LPI-neutralizing concentrations by a particular chelation treatment and the extent to which chelators remain in the circulation over time at LPI-neutralizing concentrations. This is of particular significance in view of the tendency of NTBI and LPI to rebound as serum chelator concentrations decline (Cabantchik *et al*, 2005; Porter *et al*, 2005) and remain elevated after a prolonged chelator wash-out (Piga *et al*, 2009). Yet, as this study involved a relatively small number of patients that were neither matched for baseline iron parameters nor randomized for chelation treatment, its clinical implications should be regarded as tentative rather than conclusive.

As shown in Fig 1, the mean daily or nightly LPI values were statistically elevated over the normal range in about 40% of patients treated with DFO and about 60% of those treated with DFP, respectively. This indicated considerable individual variation in the adequacy of the DFO and DFP regimens, according to the LPI criterion. Patients whose daily and/or nightly LPI values significantly exceeded a mean threshold value of 0·45 μmol/l were identifiable (Fig 2), particularly during the washout period of the drug. However, it is also noteworthy that in about 40–60% of patients undergoing prolonged (i.e. >1 year) monotherapy with either DFP daily or with DFO nightly, there was no significant rise in LPI even after the longest chelator washout period of about 10 h. Overall, combinations of DFP and DFO or monotherapy with DFR were more apparently effective in their ability to sustainably curb the daily appearance of LPI (Fig 1), consistent with their demonstrated

clinical efficacy (Kattamis *et al*, 2006; Mourad *et al*, 2003; Alymara *et al*, 2004; El-Beshlawy *et al*, 2008; Wonke *et al*, 1998; Tanner *et al*, 2007). However, even within these treatment groups individual profiling enabled the identification of patients with elevated daily or nightly LPI (1/10 on combination therapy and 2/10 on DFR), presumably caused by inadequate chelation or poor compliance (Fig 2). For patients on DFP, DFO or a combination of these, the daily LPI profiles were assumed to represent a steady state condition, as the patients had been on that therapy for at least a year and subsequent LPI testing (6–9 months later) showed essentially similar profiles. On the other hand, patients in the DFR-treated group (IV), all previously on either DFP or DFO, were subjected to the DFR regimen for the first time. However, in line with recent reports (Zanninelli *et al*, 2007; Daar *et al*, 2009), the initial (24 h) effect of DFR on daily LPI levels was sustained, with suppression of circadian LPI appearance being observed in all patients that continued the treatment for 12–24 weeks.

The tentative conclusion from our studies is that a single day LPI profile of individual patients may provide a possible means of assessing the efficacy of chelation treatment in reducing daily LPI levels, particularly in patients that have been on a fixed regimen for more than 6 months. However, in practical terms, a single determination of the trough value of LPI, namely the value after the longest period of drug washout, might be sufficiently informative and definitely more convenient than periodic daily sampling. Thus, for DFP or DFR, the early morning sample is most significant, taken just before intake of the first morning dose of chelator, whereas for DFO it is the evening sample, taken just prior to, or as close as possible to the start of the nightly infusion.

The extent to which daily LPI profiles can contribute toward improving the treatment of iron overload remains to be established by long term prospective studies addressing the correlation between LPI/NTBI levels attained at drug trough levels and the indices of liver and heart functions, as well as markers of tissue iron overload (St Pierre *et al*, 2005; Wood, 2008) including surrogate markers such as serum ferritin (Porter & Davis, 2002). As to the latter, our study based on single time point determinations, showed no quantitative, statistically significant correlation between daily or nightly levels of LPI and serum ferritin. However, an association was found between classical indicators of substantial overload (serum ferritin levels >1000 μg/l and transferrin saturation >75%) and increased mean daily or nightly LPI levels (>0·5 μmol/l). Since iron overload has been shown to lead to excess myocardial iron accumulation (Jensen, 2004), the correlation between iron overload and elevated LPI might have some pathophysiological and clinical implications. The same pertains to a recent retrospective study (Piga *et al*, 2009) in which some broad correlation was found between the appearance of cardiac complications and the presence of sustained levels (i.e. following prolonged chelator washout) of NTBI, implicating the latter as the source of cardiac iron overload.

As shown in the Figs S1–S4, the individual patients in this study showed a variable tendency to undergo resurgence in LPI levels following chelator washout. While the causes for this are a matter of conjecture, they are likely to be a function of the baseline amount of iron overload caused by intensive red blood cell clearance, but could also reflect differences in chelator metabolism and clearance. Thus, chelator choice may strongly affect the tendency for LPI to re-emerge. In the present study DFO and DFP combinations and DFR monotherapy appeared to be more effective in suppressing LPI than monotherapy with DFP or DFO, although these preliminary results will need be confirmed by larger trials. Irrespectively, it is noteworthy that excessive iron accumulation might not always be preventable by treatments aimed only at curbing excessive daily rises in LPI. Therefore, efficacious treatment might depend on the ability of chelators to access and neutralize both, the extra-cellular and intra-cellular labile iron pools (Cabantchik *et al*, 2009).

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Disclosure

ZIC and WB provided in the past scientific and technical advice on NTBI and LPI assays, as part of the Hebrew University-Yissum license to Aferrix, Tel Aviv.

Contribution of authors to the MS

Giuliana Zanninelli: study design, data acquisition and compilation, writing of MS; William Breuer: data acquision and analysis, writing of MS; Z. Ioav Cabantchi: study design, data analysis, writing of MS.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Twenty-four hour LPI monitoring of nine thalassaemia major patients treated with DFO (group I) (40 mg/kg per day given s.c. overnight).

Fig S2. Twenty-four hour LPI monitoring of nine thalassaemia major patients treated with DFP (group II) (75 mg/kg per day) given orally in three daily doses.

Fig S3. Twenty-four hour LPI monitoring of nine thalassaemia major patients treated with DFP (daily as in Fig S2) and DFO (nightly, as in Fig S1) (group III).

Fig S4. Twenty-four hour LPI monitoring of thalassaemia major patients treated with DFR (30 mg/kg per day orally) (group IV).

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