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ORIGINAL ARTICLE

Utility of Labile Plasma Iron Assay in Thalassemia Major Patients

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Abstract Labile plasma iron (LPI) levels are proposed as marker of iron overload in thalassemia patients and are also known to be the earliest parameter to indicate efficacy of chelation therapy. It was a prospective study in 35 patients of thalassemia major. Patients were recruited in two groups—group A (n = 13) patients not on chelation therapy and group B (n = 22) patients who were on regular oral chelation therapy. Ten age and gender matched healthy controls were also studied. For all patients, ferritin levels and LPI levels were measured at baseline, 6 months and 12 months. For group B patients paired samples for LPI were taken (before and 2 h after chelator). LPI levels were found to be significantly higher in group B patients versus group A patients versus normal healthy controls at all time-points. (P value—< 0.0001, 0.001) In group A, both LPI levels and ferritin levels follow an upward trend and correlated well with each other (P value—< 0.0001). In group B, the serum ferritin trend was not significant over follow up period of 1 year (P value 0.16), however LPI levels showed a significant decreasing trend on continued chelation (P value 0.0347) In patients on chelation therapy, the immediate change (2 h) in LPI levels on administration of chelators was not found to be significant (P value 0.22). LPI assay appears potentially attractive alternate to serum ferritin and can serve to monitor the trend of iron overload during long-term follow up.

Keywords Labile plasma iron · Thalassemia major · Non transferrin bound iron · Transfusion · Ferritin · Chelation

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Background

Despite steady advances in iron chelation therapy, iron mediated cardiotoxicity remains a leading cause of mortality in iron overloaded thalassemia patients [1]. The existing marker which is routinely used to measure iron overload is serum ferritin levels which has its own limitations [2]. Labile plasma iron (LPI) levels have been found to correlate well with ferritin levels in iron overloaded patients and have been found to be useful in assessing the short term efficacy of chelation [3–5]. It represents that component of non-transferrin bound iron which is capable of freely permeating into organs and deposited causing tissue iron overload [6]. Sustained higher levels of LPI over time compromise functions of vital organs (e.g. heart and liver) and affect patient survival. This has led to increasing new interest recently to measure this component in iron overloaded patients.

LPI assays have been mostly studied in thalassemia patients and at present, the research studies of LPI in thalassemia major have concentrated around it being a measure of iron overload, as measure of chelation efficiency and as surrogate marker for early cardiac damage in iron overloaded patients. Cabantchik et al. [7, 8] demonstrated the usefulness of assessing LPI levels periodically to monitor iron overload in his study on thalassemia intermedia patients. The data regarding the use of LPI to measure iron burden in transfusion dependant thalassemia major patients is limited. Due to its high redox activity, LPI is rapidly inhibited by exogenously added chelator in contrast to generally used indices of body iron stores, such as serum ferritin or transferrin saturation which respond to chelator treatment over a period of weeks to months [9–11]. Though the effect of chelators on labile plasma iron levels is well established, data regarding what cut offs of LPI levels to be used for dose escalation/change of treatment is not available. In iron overload, extrahepatic iron loading occurs through unregulated transport of non-transferrin bound iron species causing unabated tissue iron overload. T2* MRI have shown to have high predictive value to detect early iron deposits before functional impairment occurs and remains gold standard till now [12, 13]. Piga et al. [14] in their study on thalassemia major patients found that none of the patient without a high LPI or transferrin saturation below 70% had any heart symptom suggesting the possibility of LPI levels as early marker of cardiac damage.

LPI assays have been extensively studied in thalassemia patients in research settings. In this study, we attempted to explore the utility of LPI assay in practical settings and to assess whether its levels can help guide the physicians regarding chelation therapy. To the best of author's knowledge, this is the first Indian study on labile plasma iron assay in thalassemia major patients.

Methods

This was a prospective study covering a period of nineteen months (May 2016–Nov 2017) done on thalassemia major patients who visited our OPD. A total of 35 patients were recruited in two major groups:

Group A Transfusion dependent thalassemia major patients who were newly diagnosed and were not on any form of chelation therapy yet (n = 13).

Group B Transfusion dependent thalassemia major patients who were on some form of regular oral chelation (deferasirox or deferiprone or combination of both) therapy (n = 22).

LPI levels in 10 age and gender matched controls were also studied for comparison. The patients were followed up for a period of one year. Their ferritin and LPI levels were measured at baseline, 6 months and 12 months. Their clinical profile and other investigations were taken from patient's record file. For group B patients, paired samples for LPI were taken (before and 2 h after oral chelator) to assess the immediate change in LPI levels due to chelation in order to see chelation efficiency at these time points.

Patients on injectable chelation therapy and all seropositive cases were excluded from study. The study was approved by the institute's ethics committee and informed written consent was obtained in all cases. Serum ferritin was done using enzyme linked immunosorbent kits (based on principle of sandwich ELISA). For LPI assay, blood was collected in plain vial and serum was separated within 45 min. For batchwise processing, it was stored at − 70 °C. Repeated thawing and exposure of samples to direct sunlight was avoided at all times. LPI was measured by fluorescent quenching methods using FeROS LPI kit (Produced and marketed by Aferrix Ltd, Tel Aviv, Israel). The fluorescence was measured on multiplate reader in fluorescence mode using flourescin filters at 37 °C. Positive and negative standards were used in each batch as provided in the kit. The tests were done in duplicates and mean value was taken. The following cut offs were used in the study (as recommended by the kit);

LPI Negative (normal)—< 0.4 ng/mL or LPI units.

LPI low positive/borderline—0.4–0.6 ng/mL or LPI units.

LPI positive—> 0.6 ng/mL or LPI units.

For statistical analysis, quantitative variables were compared using independent T test/Mann–Whitney Test (when the data sets were not normally distributed) between the two groups and ANOVA between three groups. One sample "*t*" test was used to compare LPI and ferritin trend with test value '0'. Qualitative variables were correlated using Chi Square test/Fisher exact test. Pearson correlation coefficient was used to assess the association of various parameters with each other. P value of < 0.05 was considered statistically significant. The data was analyzed using Statistical Package for Social Sciences (SPSS) version 21.0.

Results

a. *Study population*—The details of group A and B and their clinical parameters have been outlined in Table 1.
b. Serum ferritin and labile plasma iron levels.

At baseline, the mean LPI levels in healthy controls, patients of group A and group B were 0.27 ng/mL (0.27 ± 0.1), 0.36 ng/mL (0.36 ± 0.18) and 1.83 ng/mL (1.83 ± 0.97) respectively with P value of < 0.001.

Table 1 Demographic, clinical and laboratory parameters of both the study groups

Parameter	Group A—thalassemia major patients not on any chelation therapy (n = 13)	Group B—thalassemia major patients on regular oral chelation therapy (n = 22)
Male/females (n)	9/4	16/6
Age, mean ± SD (years)	1.48 ± 0.72	9.14 ± 3.45
Splenomegaly	11 patients	21 patients
Average size of spleen (mean ± SD cms)	3.46 ± 1.29	7.24 ± 2.90
Average pretransfusion Hemoglobin, mean ± SD (gm %)	5.86 ± 1.38	6.79 ± 1.10
Deranged LFT	3 patients (23.08%)	9 patients (40.91%)
Chelation therapy	–	Deferasirox/defriprone/both—20/1/1 patients
Ferritin, mean ± SD (ng/mL)		
Baseline	256.17 (256.17 ± 170.73)	2570.77 (2570.77 ± 1864.01)
6 months	344.46 (344.46 ± 210.46)	2349.25 (2349.25 ± 1638.74)
12 months	435.27 (435.27 ± 155.24)	2273.22 (2273.22 ± 2133.85)
LPI levels, mean ± SD (ng/mL)		
Baseline	0.36 (0.36 ± 0.18)	*1.83 (1.83 ± 0.97)
6 months	0.40 (0.40 ± 0.19)	*1.64 (1.64 ± 0.84)
12 months	0.46 (0.46 ± 0.16)	*1.58 (1.58 ± 1.12)

*Mean LPI 1 levels (before Chelator)

In group A, both ferritin and labile plasma iron levels correlated with each other and showed an upwards trend during the follow-up period as shown in Figs. 1 and 2 (P value < 0.0001, P value—0.001). In group B, the mean level of labile plasma iron level before oral chelator (i.e. LPI 1) at three time points were 1.83 ng/mL (1.83 ± 0.97), 1.64 ng/mL (1.64 ± 0.84) and 1.58 ng/mL (1.58 ± 1.12) respectively. The LPI levels showed a downwards trend over 1 year (P value of 0.0347) follow-up while ferritin did not show a significant down trend (P value—0.16) (Figs. 1, 2). The mean LPI levels post chelator administration (i.e. LPI 2) was 1.68 ng/mL (baseline), 1.49 ng/mL (6 months) and 1.49 ng/mL(12 months). The average fall of LPI levels

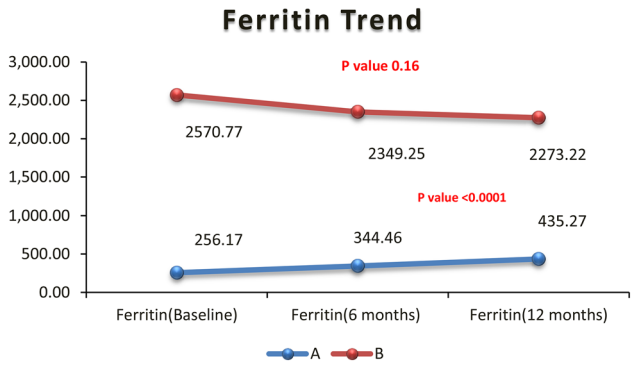

Fig. 1 Ferritin levels in Group A and B at baseline, 6 and 12 months. Group A shows a significant (P value < 0.0001) upwards trend of ferritin while in group B the trend was not found to be significant (P value 0.16)

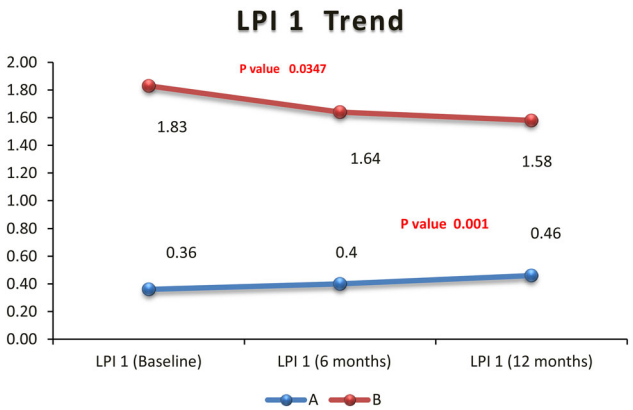

Fig. 2 Mean LPI levels in Group A and B (LPI1) at baseline, 6 and 12 months. Group A shows a significant (P value 0.001) increasing trend of LPI (matching with ferritin) while in group B, LPI levels showed a significant downwards trend (P value 0.0347) over one year when ferritin trend was not found to be significant (P value 0.16)

(LPI 2–LPI 1) 2 h post chelator in group B was 0.15, 0.05 and 0.09 at three time points which was not found to be significant at any timepoint (P value 0.22) as shown in Fig. 3.

c. Correlation of labile plasma iron with other parameters.

We found correlation of mean LPI levels with some clinical parameters like increasing age of patients (P value < 0.0001, Pearson's correlation coefficient 0.647)

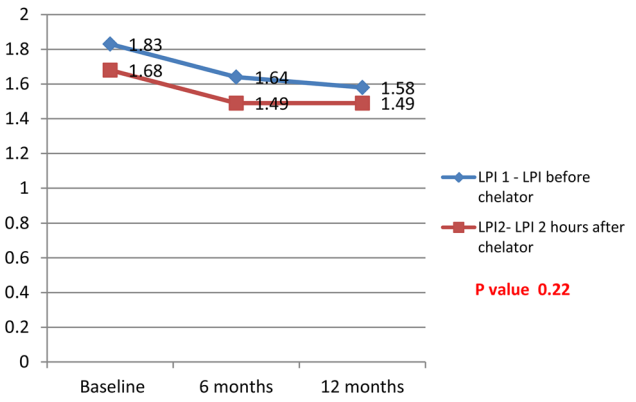

Fig. 3 Fall in LPI levels (LPI 2–LPI1) 2 h after administration of chelator at baseline, 6 months and 12 months in patients of group B

and splenomegaly (*P* value 0.004, correlation coefficient 0.49); but no correlation was found between LPI levels and frequency of transfusion (*P* value 0.246, correlation coefficient; − 0.201) or liver function tests i.e. serum bilirubin and liver enzymes (*P* value 0.463, correlation coefficient; − 0.391). Information about other markers of iron overload like transferrin saturation (TSAT) and T2*cardiac MRI were available only in few patients of group B (08 and 04 patients respectively). We did not find any significant correlation between LPI levels (both LPI 1 and LPI 2) with TSAT (*P* value 0.929, correlation coefficient; − 0.038) or with T 2* cardiac MRI findings (*P* value 0.521, correlation coefficient − 0.479).

Discussion

This study was undertaken to assess the utility of LPI levels in clinical settings in management of transfusion dependant thalassemia major patients. We studied both thalassemia major patients not on chelation (group A)and on chelation therapy (group B). The mean age of patients in group A was higher than group B because of our selection criteria (usually the chelation is started in patients by the age of 2–2.5 years). The age of onset of symptoms in the study group was 6.25 months which correlates well with established literature [15]. All 35 patients in the study cohort were symptomatic with anemia requiring regular transfusions ranging from fortnightly to two monthly. (group A vs. group B—*P* value 0.062). The patients in Group B had significantly larger spleen (*P* value 0.003) and lower average pretransfusion haemoglobin (*P* value—0.03) due to longer duration of disease in this group. The mean age of starting chelation therapy in the group B was 3.54 years and a large majority of the patients (81.82%) were irregular with their chelation therapy before inclusion in the study.

Ferritin Levels

Group A patients had normal or near normal serum ferritin at baseline which persistently increased on follow up due to accumulation of transfusional iron. The mean ferritin levels in group B patients (on oral chelation therapy) was significantly higher than the patients in group A at all the time points (*P* value < 0.0001, 0.0001 and 0.002 respectively) which can be attributed to longer duration of disease and more number of transfusions in this group.

An important observation in our study was that while the ferritin trend of patients in group A was significant (upwards trend and *P* value < 0.0001), the ferritin trend in patients of group B did not come out to be significant (*P* value 0.16) on 1 year follow up indicating that at later stages of the disease the ferritin changes are not sensitive to increasing burden of tissue iron overload. This observation is in concordance with findings of Adamkiewicz et al. [16] wherein they concluded that a lack of fall in ferritin levels with chelation does not necessarily prove that the patient is a 'non responder' to the chelation regime. This could be because the relationship between body iron and ferritin is not always linear, particularly in the context of inflammation tissue damage or late stages of disease [16]. Davis et al. [17] in their study on thalassemia major patients demonstrated that below 3000 µg/L ferritin values are influenced mainly by iron stores in the macrophage system, whereas above 3000 µg/L they are determined increasingly by ferritin leakage from hepatocytes.

LPI Levels

We found mean LPI levels in thalassemia major patients to be significantly higher than in healthy controls even for newly diagnosed cases at baseline *P* value of < 0.001) which could be due to increased absorption of iron from intestine in patients of thalassemia. In group A, patients showed increasing LPI levels from baseline to 1 year on follow-up which correlated very well with increasing ferritin (*P* value 0.001) in group A.

The mean LPI1 levels (i.e. LPI levels before chelator) in group B was significantly higher than the patients in group A at all the time points (*P* value < 0.0001, < 0.0001 and < 0.0001 respectively). However, in patients of group B, while the ferritin trend over 1 year follow up was insignificant (*P* value 0.16) the LPI trend showed a significant decreasing trend from baseline to 1 year (*P* value 0.0347) on follow up indicating that LPI levels are more sensitive to chelation therapy (thereby better indicator of tissue iron load) as compared to serum ferritin. Study by Cabantchik et al. demonstrated LPI levels to be significantly higher in thalassemia patients on chelation as compared to controls (controls—0.20 ng/mL, thalassemia

patients on chelation, baseline LPI—0.80 ng/mL) [11]. However, they did not include any chelation naïve patients in their study [7, 8]. Zanninelli et al. [4] also demonstrated that LPI levels to be higher in thalassemia patients and that these assays are better suited for measurements when iron chelators are present in the plasma.

In group B patients paired samples for LPI were taken (LPI 1—before chelator and LPI 2—2 h after chelator) to assess the immediate change in LPI levels due to chelation in order to see chelation efficiency. However, we did not find significant change/fall in LPI levels (LPI 2–LPI 1) post chelator administration at any timepoint (*P* value 0.22). This finding is in contrast with the findings of previous studies by Pootrakul et al. and Alymara [8, 18] wherein they demonstrated that being the redox active component of plasma, LPI activity is rapidly inhibited by exogenously added chelator and is the earliest measurable parameter affected by chelator ingress into body fluids. Zanninelli et al. [4] demonstrated a significant fall of LPI levels (before chelator—0.95 ng/mL, 2 h post chelator—0.16 ng/mL *P* value 0.023) post administration of chelators by daily measurement of LPI levels and concluded that detectable levels of LPI post chelator is an indicator of ineffective chelation. The authors proposed that daily measurement of LPI levels can help assessing the early effectiveness of chelation protocol [4]. In our study, since we did not find a significant fall of LPI level 2 h post chelation at any time points of measurements, such inference cannot be made.

LPI Assay—The LPI assay by fluorescence is convenient and procedure is fairly simple. The test can be performed batchwise with proper storage of serum at appropriate temperatures. However, exposure to direct sunlight and high temperature at any step can lead to fallacious results. Also, requirement of a multiplate fluorescence reader may be a limitation in few settings.

Limitations of the Study

Due to small sample size, the findings of our study may be at variance with other similar studies. We could not perform daily LPI levels in patients on chelation which would have served as more appropriate indicator of chelation efficiency. The follow-up period of one year is too short to comment on cardiac damage in patients of thalassemia. Also, correlation with T2* cardiac MRI and Liver iron concentration would have added more information to the study.

Conclusion

Iron overload is inevitable in thalassemia patients and a single ideal test to measure the same is yet to be discovered. Labile plasma iron assay appears potentially attractive candidate and can serve as an alternate to serum ferritin in cases where ferritin appears to be fallacious (particularly with deranged liver function). It may also serve to monitor the trend of tissue iron overload during long-term follow up where they're better than ferritin levels. However, the extent to which LPI profiles can contribute towards assessing chelation efficiency remains to be established by long term prospective studies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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