Timed non-transferrin bound iron determinations probe the origin of chelatable iron pools during deferiprone regimens and predict chelation response

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Plasma non-transferrin bound iron refers to heterogeneous plasma iron species, not bound to transferrin, which appear in conditions of iron overload and ineffective erythropoiesis. The clinical utility of non-transferrin bound iron in predicting complications from iron overload, or response to chelation therapy remains unproven. We undertook carefully timed measurements of non-transferrin bound iron to explore the origin of chelatable iron and to predict clinical response to deferiprone.

Design and Methods

Non-transferrin bound iron levels were determined at baseline and after 1 week of chelation in 32 patients with thalassemia major receiving deferiprone alone, desferrioxamine alone, or a combination of the two chelators. Samples were taken at baseline, following a 2-week washout without chelation, and after 1 week of chelation, this last sample being taken 10 hours after the previous evening dose of deferiprone and, in those receiving desferrioxamine, 24 hours after cessation of the overnight subcutaneous infusion. Absolute or relative non-transferrin bound iron levels were related to transfusional iron loading rates, liver iron concentration, 24-hour urine iron and response to chelation therapy over the subsequent year.

Results

Changes in non-transferrin bound iron at week 1 were correlated positively with baseline liver iron, and inversely with transfusional iron loading rates, with deferiprone-containing regimens but not with desferrioxamine monotherapy. Changes in week 1 non-transferrin bound iron were also directly proportional to the plasma concentration of deferiprone-iron complexes and correlated significantly with urine iron excretion and with changes in liver iron concentration over the next 12 months.

Conclusions

The widely used assay chosen for this study detects both endogenous non-transferrin bound iron and the iron complexes of deferiprone. The week 1 increments reflect chelatable iron derived both from liver stores and from red cell catabolism. These increments correlate with urinary iron excretion and the change in liver iron concentration over the subsequent year thus predicting response to deferiprone-containing chelation regimes. *This clinical study was registered at clinical.trials.gov with the number NCT00350662.*

Key words: non-transferrin bound iron, iron chelation, thalassemia major.

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Introduction

Classic studies, using radioactive iron probes in iron-overloaded animals or humans, showed that chelatable iron is derived mainly from two pools; one from ferritin catabolism in hepatocytes and the other from red cell degradation in macrophages.[1-5] Iron mobilized by chelation is excreted as iron-chelate complexes in urine and feces with desferrioxamine (DFO) and mainly in urine with deferiprone (DFP).[6-8] With chelation regimens that include both DFP and DFO both fecal and urinary iron excretion occur.[9] Iron-chelate complexes of DFP are likely to be present in the plasma in patients treated with DFP containing-regimens, but the relationship of their plasma concentration to measures of body iron stores or iron turnover has not been reported.

The term 'plasma non-transferrin bound iron (NTBI)' refers collectively to any plasma iron species that is not bound to transferrin.[10] These include a variety of iron-citrate and protein-bound species,[11-13] but in principle could also include the iron complexes of some chelators during chelation regimens. The most widely performed NTBI assay uses nitrilotriacetate to capture NTBI species, followed by a detection step using high performance liquid chromatography[14-16] or ferrozine.[17,18] Alternatively NTBI can be estimated indirectly by measuring the redox activity of plasma samples in the labile plasma iron assay.[19] We have shown that the iron complex of the hexadentate DFO is not detected using the nitrilotriacetate-NTBI assay[15,20] but the less stable iron complexes of the bidentate DFP[20], or tridentate deferasirox[21] or even the novel tridentate oral chelator FBS0701, a desferrithiocin analog currently undergoing clinical evaluation, are potentially detectable with this assay. With DFP-containing regimens, the iron free-chelator is rapidly metabolized and eliminated with a short half-life of about 77 to 91 minutes.[7,22] However data on the kinetics of chelate-complex elimination are lacking and in principle these complexes may be released from cells and detectable in plasma, even after the free ligand has been eliminated.

In this study we took blood samples 10 h after an evening dose of DFP, a sampling time when free ligand of DFP would have been eliminated, but when the complex may still have been present in the plasma. We assayed NTBI under these conditions at quarterly intervals after commencing chelation with a DFP-containing regimen and compared NTBI values to baseline, prior to starting DFP. NTBI values were then related to the baseline liver iron concentration (LIC), the transfusional iron loading rates, the 24 h urine iron and the concentration of DFP complexes in the plasma in order to gain insight into chelatable iron pools and NTBI. Furthermore, by following patients for 1 year, and repeating LIC measurements at this time, we have been able to explore the predictive values of such NTBI measurements on LIC response and hence iron balance at 1 year.

Design and Methods

Study design

This was an NTBI sub-study in patients randomized into a trial that compared responses of ferritin, LIC and 24 h urine iron to DFP given either as monotherapy or combined with DFO twice weekly, as previously reported.[9] Chelation doses were as follows: DFP (LIPOMED AG, Switzerland) was given at a total daily dose of 75 mg/kg in three divided doses (at 8 am, 3 pm and 11 pm) either alone or in combination with DFO (40-50 mg/kg sc, twice weekly, always on the same 2 consecutive nights) given as a night-time infusion between 10 pm and 9 am. In patients on combination therapy, two of the three daily DFP doses were administered simultaneously with the DFO infusion (at 11 pm and 8 am). A control group of 12 patients on DFO monotherapy were included in order to determine whether any observed NTBI changes were independent of DFP chelation. All patients had been treated with DFO prior to the study and had a wash-out phase without any iron-chelating medication for 2 weeks before initiation of treatment. Blood sampling for NTBI measurements and 24 h urine collection for urinary iron excretion (UIE) assays were performed at baseline prior to transfusion and after 1 week of chelation. The patients receiving combination therapy collected urine on 2 days, during 1 day of DFP monotherapy and on the second day of the combination treatment.

Patients

Twenty patients with thalassemia major received a DFP-containing regimen for 1 year: 12 received daily DFP monotherapy while eight received additional DFO twice a week overnight (see above). A further 12 patients received DFO monotherapy to obtain control information about NTBI changes in the absence of DFP treatment. The baseline characteristics assessed in the patients receiving DFP-containing regimens (Table 1) are as previously described and included serum ferritin, transferrin saturation, LIC, transfusional iron loading rates and UIE.[9] Twelve patients were originally recruited in the combination treatment arm, but four dropped out of the study: two patients withdrew their informed consent just after enrollment because they refused DFO therapy, one died from arrhythmia-induced congestive heart failure just at the beginning on day 7 of the study and one developed agranulocytosis at week 14. All patients received regular blood transfusions at intervals of 2–4 weeks with mean pre-transfusional hemoglobin ± standard deviation (SD): 9.1±0.47 g/dL. This study was approved by the Institutional Review Board of the Ministry of Health of Turkey and the local ethics committee and all patients treated in this study gave written informed consent prior to entering the study.

Plasma non-transferrin bound iron

Baseline blood samples for NTBI measurements were taken after a 2-week washout period without chelation, at 9 am pre-transfusion. In patients on combination therapy, two of the three daily DFP doses were administered simultaneously with the DFO infusion (at 11 pm and 8 am). Samples for NTBI measurements were also obtained 1 week later and at quarterly intervals

Table 1. Baseline characteristics of the patients.

	DFP mean±SD	DFP+DFO mean±SD	DFO mean±SD
Age (years)	15.9±4.3	17.4±5.0	17.3±0.57
Pre-transfusional Hb (g/dL)	9.0±0.36	9.2±0.39	9. 2±0.52
Ferritin baseline (μg/L)	4070±3230	4060±3379	2905±2519
LIC baseline (mg/g dry wt)	30.7±10.6	26.6±15.4	18.7±0.86
Transferrin saturation (%)	96.0±10.7	94.9±6.64	96.3±7.41
Transfusional iron loading rate (mg/kg/day)	0.30±0.06	0.27±0.07	0.34±0.14
UIE baseline (mg/kg/day)	0.017±0.017	0.011±0.013	0.002±0.002

thereafter, taken in each case in the morning, 10 h after the previous evening's dose of DFP. For those patients on combination therapy or on DFO monotherapy, the sample for NTBI measurements was taken 24 h after the last DFO infusion had been completed. Venous blood was withdrawn with metal-free needles into vacutainer tubes which had been tested for iron contamination and contained $AlCl_3$ solution (20 mM, 10 μL/mL of blood). Aliquots of serum were made in cryo-tubes which were then stored at -80°C. The method used for determining NTBI is that of Singh *et al.*[14] This method is based on mobilization of NTBI with nitrilotriacetate which is added to the serum at a high concentration and acts as a 'gathering' ligand for iron in the various sub-fractions of NTBI. The iron-nitrilotriacetate complex is subsequently filtered through 30 kDa filtration devices and quantified by on-column derivatization with 3-hydroxy-1-propyl-2-methyl-pyridin-4-one (CP22) in a metal-free Waters 625 LC system equipped with a 996 photodiode array detector (Waters Ltd.).

Deferiprone iron complexes

DFP iron complexes were determined in week-1 serum samples from patients on DFP-containing regimens. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (Chaps 20 μL, 100 mM) was added to 180 μL of serum and the mixture incubated at room temperature for 10 min. The treated serum was then centrifuged through a 30 kDa filtration device (VWR UK) to remove proteins of higher molecular mass. Under these conditions the DFP-iron complex filters completely. Fifty microliters of the filtrates were injected into a 5 μm microsorb C18 column (Agilent technologies CP914915) equilibrated to 5 mM MOPs buffer at pH 7.8 containing 5% acetonitrile. DFP-iron complexes were detected at 460 nm using the above described Waters 625 LC system and quantified by injection of standard solutions of the complexes prepared at a ratio of DFP ligand to iron of 3:1. Since plasma complexes of DFP are predicted to have a 3:1 stoichiometry of ligand to iron, this ratio was used in the standards so that any tendency to complex dissociation during the high performance liquid chromatography would occur to the same extent in the samples and standards used. The high linearity of the standard curve used for the calculation of complex concentration in serum attests to this (R^2=0.99).

Liver iron concentration

LIC was assessed in biopsies obtained from one pass of a Menghini type biopsy needle in all patients. The assessment of LIC was completed within 4 weeks prior to study in all patients. Liver biopsy samples of less than 0.5 mg dry weight were excluded from analysis. Fresh biopsy samples were stored frozen at -80° C prior to analysis. Iron was measured by inductively coupled plasma - atomic emission spectrophotometry (ICP-AES) using a Jobin Yvon JY spectrophotometer and expressed as mg/g dry weight.[23] LIC was also assessed in the same type of biopsy after 1 year of treatment.

Twenty-four-hour urinary iron excretion

Urine was collected for 24 h at baseline after a 2-week washout period without chelation, prior to transfusion and at week 1 to measure UIE. For patients receiving DFO alone, 24 h urine samples were taken from the beginning of the DFO infusion until the same time 24 h later. For patients receiving DFP, 24 h urine samples were taken from the first DFP dose in the morning until the same time 24 h later. For patients receiving combination therapy, two 24 h urine collections were made: (i) for exactly 24 h on any of the days of DFP single treatment and (ii) on the second of the two consecutive weekly DFO doses. The mean 24 h urine iron for the week in question was then calculated from the equation (5 x a + 2 x b)/7. Urinary iron was measured by ICP-AES using a Jobin Yvon JY spectrophotometer.[23] The average UIE of quarterly measurements during 1 year of study was calculated for each patient and expressed as mg iron /kg/day.

Iron balance

The patient's iron balance was derived from the change in LIC over 1 year of treatment and is expressed as the mean change in body iron per kg body weight per day, calculated using the following formula: change in body iron (iron balance) in mg/day = [(LIC at T_0 - LIC at T_{1y}) x 10.6 x body weight in kg] / number of days on treatment between biopsies, as previously described by Angelucci *et al.*[24]

Transfusional iron loading rate

This was calculated from the blood volume transfused between baseline and the end of the study. The average iron content per transfusion unit, derived from the measured hematocrit, was 154 mg. The transfusional iron loading rate was then expressed in mg of the transfused iron per kg body weight per day.

Statistical analyses

Results are expressed as the mean ± SD, unless otherwise stated. Differences between means were tested using the unpaired, one-tailed t-test, unless otherwise stated. Pearson's correlation coefficients were used to examine how variables were related.

Results

Baseline characteristics of the patients

Table 1 presents the characteristics of the 12 patients in the DFP arm, eight patients in the combination arm and an additional control group of 12 patients treated with DFO monotherapy. Pre-transfusional hemoglobin and transfusional iron loading rate values shown are those for the year of the study. The mean age, pre-transfusion hemoglobin, baseline serum ferritin levels and LIC were comparable between the DFP monotherapy and DFP+DFO combination treatment groups (Table 1). Baseline mean LIC and ferritin values were somewhat lower in the DFO monotherapy group, indicating that patients who were additionally selected into the control group seemed to comply better with chelation therapy. UIE at baseline, following the 2-week washout, was typically less than 1 mg/day (Table 1). Transfusional iron loading rates are in line with those previously quoted for patients with thalassemia major and in the low to moderate range.[25] Baseline NTBI values correlated significantly with transferrin saturation (r=0.77, *P*=0.0001) but this relationship was lost after commencement of treatment (r=0.125, *P*=0.61).

Table 2. Change in NTBI at week 1 in each treatment group.

	N.	Baseline NTBI* mean ± SD	Week 1 NTBI* mean ± SD	Mean difference	*P*
DFP	12	4.095±1.11	5.26±2.40	1.17±1.47	0.030
DFP + DFO	8	4.17±0.69	5.76±1.69	1.62±1.50	0.024
All DFP regimens	20	4.12±1.11	5.47±1.32	1.28±1.45	0.001
DFO only	12	3.91±0.77	3.81±0.89	- 0.098±0.855	0.780

* *in μM.*

Non-transferrin bound iron changes from baseline to week 1 after commencing chelation therapy

Table 2 shows the baseline NTBI, week 1 NTBI and change in week 1 NTBI as mean ± SD for each treatment group. After 1 week of treatment, there was a significant increase in NTBI from baseline in patients treated in the DFP and combination arms (Table 2, Figure 1A), which declined slowly in the subsequent quarterly analyses (*data not shown*). This peak increase was not seen in patients who received DFO monotherapy (Table 2).

Relationship of week-1 non-transferrin bound iron to baseline liver iron concentration and transfusional iron-loading rate in patients receiving deferiprone-containing regimens

Plasma NTBI levels at week 1 (both absolute and change relative to baseline) were significantly correlated with baseline LIC (Figure 1B). NTBI levels were also inversely proportional to the transfusional iron-loading rate, showing a linear correlation with 1/transfusional iron loading rate (Figure 1C). It can be seen in Figure 1B and 1C that there was no systematic difference between these relationships for patients on DFP monotherapy (circles) and combination therapy (triangles).

Relationship of urinary iron excretion to week-1 non-transferrin bound iron, liver iron concentration and transfusional iron-loading rate in patients receiving deferiprone-containing regimens

UIE at week 1 (shown as the difference from baseline excretion) was significantly and linearly related to NTBI increments from baseline at week 1 (Figure 2A) and to 1/transfusional iron loading rate (Figure 2C). Relationships of UIE with baseline LIC (Figure 2B) did not reach statistical significance unlike those for NTBI with LIC (Figure 1B).

Relationship between non-transferrin bound iron and concentration of deferiprone-iron complexes at week 1

DFP-iron complexes were detected in week-1 sera from patients using DFP-containing regimens despite the

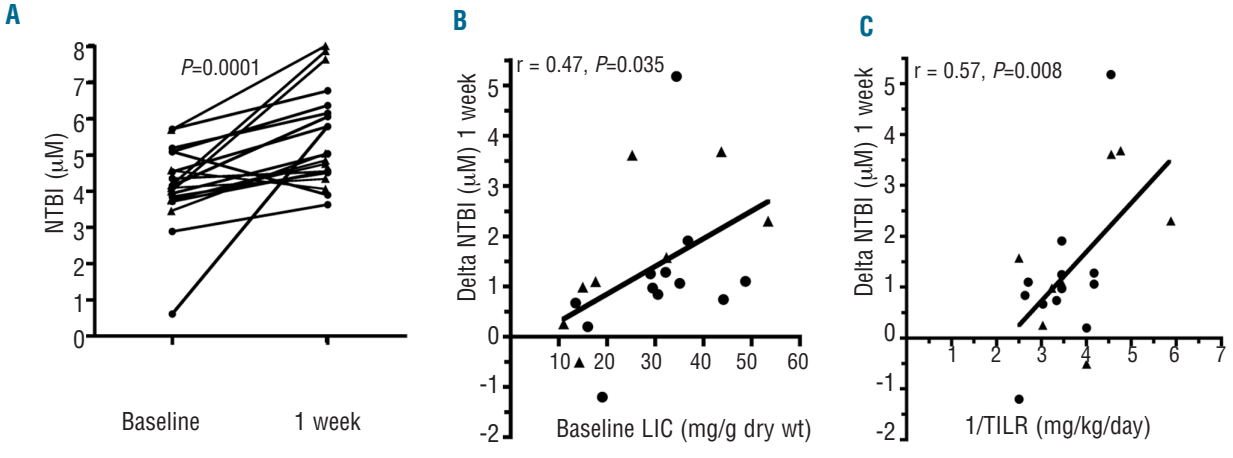

Figure 1. (A) The NTBI at week 1 is shown in relation to baseline as mean SEM. The change in NTBI at week 1 is shown in relation to; (B) baseline LIC and (C) 1/ transfusional iron loading rate (1/TILR) for patients receiving DFP regimens. Patients receiving DFP monotherapy are represented by circles and those receiving DFP in combination with DFO by triangles. The correlations shown are those for all patients.

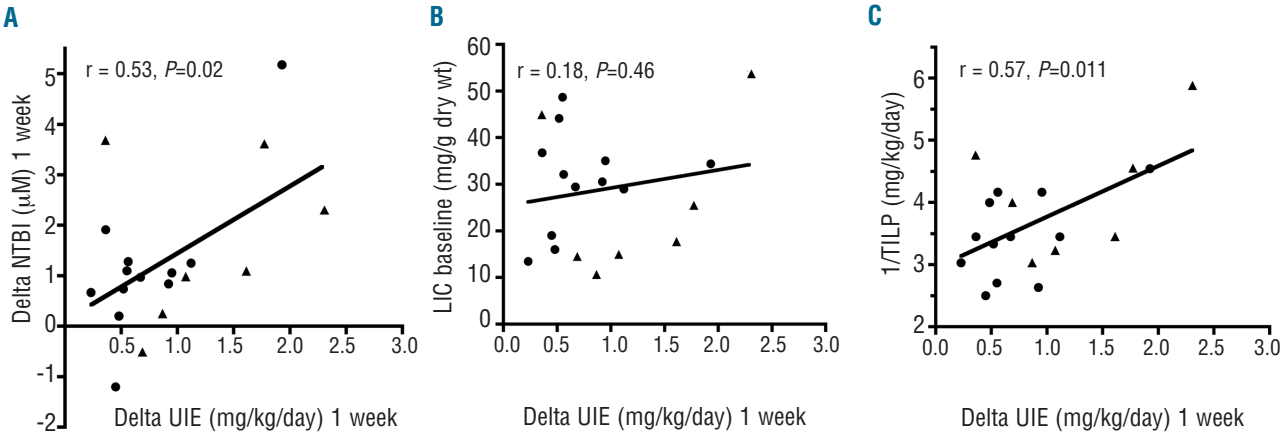

Figure 2. The relationship of the 24 h UIE measured after 1 week of therapy to: (A) week 1 change in NTBI, (B) baseline LIC and (C) 1/ transfusional iron loading rate (1/TIRL) are shown for patients receiving DFP. Patients receiving DFP monotherapy are represented by circles and those receiving DFP in combination with DFO by triangles. The correlations shown are for all patients.

absence of DFP chelation for 10 h. These complexes were tested for authenticity by co-elution of spectrally identical standard complexes prepared at a 3:1 ratio of DFP to iron (see *Online Supplementary Figure S1*). The week 1 increment in NTBI from baseline was directly proportional to complex concentration (Figure 3).

Relationship of week-1 non-transferrin bound iron to liver iron concentration changes at 1 year in patients receiving deferiprone-containing regimens

The net change in LIC over a period of time reflects iron balance and can be used to calculate this value.[25] Mean decrements in LIC over 1 year in the DFP and DFO+DFP groups were 2.1±9.3 mg/g dry weight and 8.5±9.1 mg/g dry weight, respectively. Iron balance, calculated from this change in LIC and expressed in mg/kg/day in the DFP and DFO+DFP groups was 0.06±0.26 and 0.24±0.25 mg/kg/day, respectively. There was a significant correlation (Figure 4A) between the change in LIC at 1 year and the change in NTBI at week 1 (r = 0.52, *P*=0.019); larger increases in NTBI between baseline and week 1 were significantly associated with negative iron balance at 1 year, whereas lower increments were associated with positive iron balance (net iron accumulation). Specifically, NTBI increments above 1.075 μM gave an average odds ratio of 21 (95% CI 1.78 to 248) for liver iron decrease, as opposed to increase, at 12 months (Fisher's exact test *P*=0.0198). This suggests that week 1 NTBI increments may be a useful predictor of response to DFP-containing regimens. Baseline LIC did not predict subsequent response as there was only a weak, non-significant relationship between baseline LIC and 1-year LIC (r=0.35, *P*=0.09, *data not shown*). Absolute UIE at week 1 showed a weak, non-significant correlation with change in LIC over 1 year (r=0.39, *P*=0.06, *data not shown*). However, the change in UIE at week 1 from baseline (requiring two urine measurements), showed a significant relationship to the change in LIC (r=0.54, *P*=0.017; Figure 4B).

Discussion

NTBI is assumed to be the source of catalytically active iron and can appear in the plasma even with less than full saturation of transferrin. Knowledge about the origin of chelatable iron with DFP treatment, alone or when combined with DFO, is relatively limited. We hypothesized that changes in plasma iron species such as NTBI may be proportional to the magnitude of chelatable iron pools during chelation therapy.

The increase in NTBI from baseline after 1 week of chelation with DFP-containing regimens (Table 2, Figure 1A) was unexpected and has not been previously reported. We, therefore, investigated what factors might contribute to this increase. First we found that the increase was significantly correlated with the baseline LIC (Figure 1B). We then investigated whether the transfusional iron-loading rate affected the NTBI increment with chelation therapy at week 1. Again, to our initial surprise, we found that NTBI (both absolute and incremental) after 1 week of DFP chelation treatment was greatest in patients with the lowest transfusional iron-loading rate (Figure 1C).

Since liver iron and red cell catabolism are the major

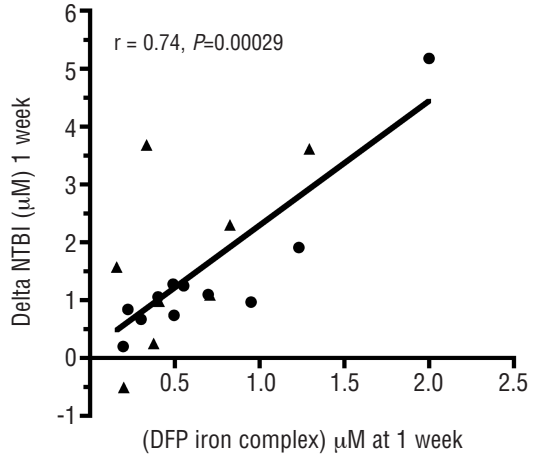

Figure 3. Change in NTBI relative to baseline is proportional to the plasma DFP-iron complex concentration at week 1. Patients receiving DFP monotherapy are represented by circles and those receiving DFP in combination with DFO by triangles. The correlations shown are those for all patients.

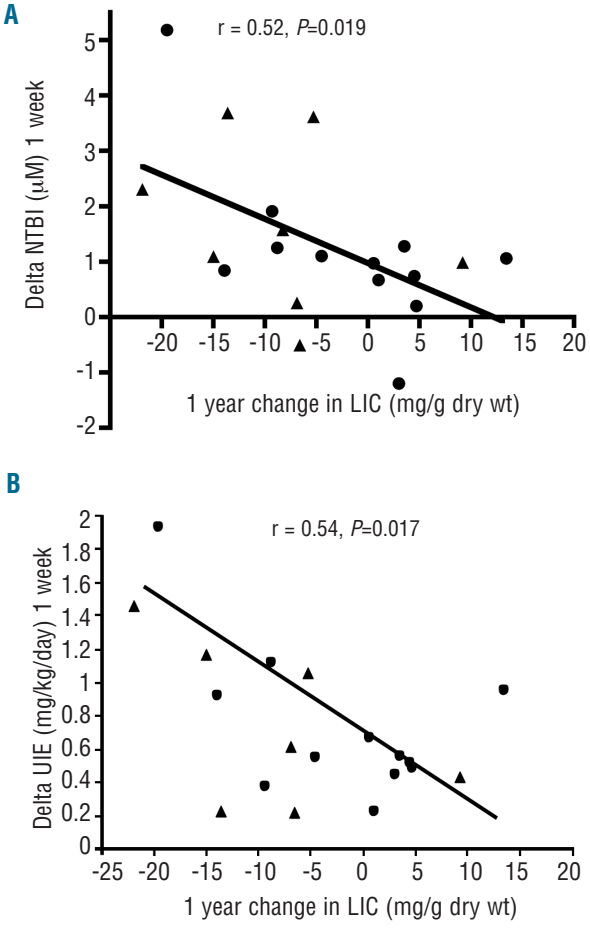

Figure 4. The change in LIC after 1 year of treatment is compared with the change in NTBI after 1 week of treatment (A) or with UIE at week 1 (B). Patients receiving DFP monotherapy are represented by circles and those receiving combination therapy by triangles. The correlations shown are for all patients.

sources of chelatable iron, we postulated that this NTBI increment reflected the major combined chelatable iron pools. If this interpretation is correct, then this timed NTBI measurement should also be proportional to the iron excreted by chelation. With DFP, nearly all chelatable iron is thought to be excreted in the urine[7,8] and we found a clear relationship between the week 1 NTBI and the increase in UIE with this treatment (Figure 2A). These findings are consistent with the week 1 NTBI increments being proportional to the available chelatable iron pools.

We then wanted to understand why timed NTBI measurements should reflect chelatable and excreted body iron pools. One possible explanation for these relationships would be that the increased NTBI at the times measured is a 'rebound' phenomenon that was first reported after ending DFO infusions.[15] In this scenario, the rebound would be greatest in patients with the highest iron turnover rate. Thalassemia major patients receiving the lowest rate of transfusion have the highest degree of endogenous erythropoiesis and, as this is largely ineffective, these patients will have the highest iron turnover rate. Hence the inverse relationship of transfusional iron loading rate with the week 1 NTBI would be consistent with this hypothesis.

An alternative explanation is that the NTBI assay is detecting the complexes of DFP as well as the NTBI present. The concentration of these complexes in the plasma would then be proportional to the summation of the chelatable iron pools and indeed to iron excreted in the urine. In order to investigate whether the second hypothesis is true, we measured the complexes of DFP in the serum samples at week 1 and indeed found that the week 1 NTBI increments were proportional to the levels of the DFP-iron complexes (Figure 3). This clear finding shows that a source of the NTBI increment is the detection of chelate–iron complexes by the NTBI assay. This is of interest because it provides evidence for the contribution of two major chelatable iron pools in thalassemia major patients receiving DFP alone or in combination with DFO and suggests that liver and red cell turnover both contribute approximately equally to the chelatable iron pools. We conclude that the increase in week 1 NTBI in our study was most likely due to the detection of chelate complexes in plasma and was critically dependent on the interval between the previous DFP dose and the taking of the NTBI sample as well as the transfusion status of the patient. The kinetics of elimination of the DFP-iron complex have not been described previously but our studies show clearly that iron complexes of DFP are present at 10 h after the evening dose and that these are detectable in the NTBI assay. This contrasts with the rapid elimination of the free DFP ligand and is likely due to a much slower release of the iron complex from cells.

A rebound in NTBI (measured using the labile plasma iron assay) after short-term administration of DFP in thalassemia major patients has been reported previously[26,27] but these studies did not attempt to link such increments to iron stores or to iron turnover. Another earlier publication regarding ten patients with thalassemia major, in which the NTBI assay used was the same as that in our study, reported a decrease in NTBI from baseline at 12 h after the last DFP dosing.[28] This may be because the washout period for baseline NTBI sampling was not sufficient (this was not stated) or because only data from later time points of 3 and 6 months after starting DFP were reported rather than those for 1 week. However, our findings show that NTBI remains increased although not at peak levels 10 hours after DFP dosing even at 6 and 12 months (*data not shown*). In another study, 17 untransfused E-β thalassemia patients showed long-term decrements of labile plasma iron during treatment with DFP.[29] The authors noted that serum samples were obtained in the morning, at least 10 h after the last dose of DFP had been taken but changes at time-points before 2 months of therapy were not reported. These untransfused patients also showed a particularly substantial reduction in iron overload at these time points, which may have contributed to the long-term decrease in labile plasma iron. However, our results show clearly that, in thalassemia major patients, NTBI increments are detectable following 1 week of therapy 10 h after dosing, and that the iron complexes of DFP contribute to this effect.

Since the week 1 NTBI appeared to be proportional to both the chelatable iron pools and the UIE, we wanted to see whether this week 1 NTBI measure might be practically useful in predicting the response of individual patients to chelation therapy. Our results suggest that this is indeed the case (Figure 4A). By comparing the week 1 NTBI with the change in LIC over the subsequent year, it can be seen that the patients who showed the largest decrease in LIC (and hence negative iron balance) were those who had the largest increase in NTBI at week 1. With DFP monotherapy two urine collections made at baseline and at week 1 also identify responders. Additional urine collections are required if patients are receiving combined treatment with DFO. An advantage of measuring blood NTBI rather than 24 h urine collections to predict response is that 24 h urine collections, outside of the context of a clinical trial, are more laborious for patients than a blood test and are often incompletely collected. Our data also suggest that UIE needs to be collected both at baseline and after treatment to obtain the clearest picture (Figure 4B). Hence plasma NTBI measured 10-12 h after an evening dose of DFP will be highest in patients who show the greatest response to therapy. We suggest that this approach might be particularly useful for chelation with deferasirox or the novel chelator FBS0701,[30] with which there is little or no UIE but with which the iron-complex is likely to be detectable in the NTBI assay used in this study. A practical question is whether it is necessary to measure the change (Δ) in NTBI from baseline (which requires two blood tests), or whether a single week 1 NTBI is adequate. Our findings show that both absolute and ΔNTBI correlated with ΔUIE (P=0.034, P=0.02) and baseline LIC (P=0.015, P=0.035), respectively. This is probably because the average magnitude of the NTBI increment is approximately one third of the baseline, so that relationships are seen even without subtracting the baseline value. However, if this test is to be used to predict LIC response in individual patients, our findings suggest that ΔNTBI at week 1 would be a more robust approach than week 1 NTBI (P=0.019, P=0.21 respectively). Further work is indicated to determine whether other assays of plasma iron species, such as an assay of the labile pool iron, or assays designed to specifically measure the plasma concentrations of the iron chelators in question, show the same relationships to those that we have identified with DFP using the classic nitrilotriacetate-NTBI assay.

In conclusion, this is the first study to link timed early changes in NTBI to the magnitude of chelatable iron pools and with response to chelation therapy. The NTBI assay used in this study, which has been widely applied in previ-

ous studies, detects both endogenous NTBI and the chelate complexes of DFP, even 10 hours after the preceding DFP dose. Our results suggest that this timed measurement is a potentially useful approach that merits further investigation both with DFP-containing regimens and with other chelation regimens in which the iron complex is likely to be detected by the NTBI assay, such as deferasirox and the novel chelator FBS0701.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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