β-Thalassemia intermedia: morbidity uncovered

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Chapter 3

Iron Overload

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Levels of non-transferrin-bound iron as an index of iron overload in patients with thalassaemia intermedia

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Summary

Non-transferrin-bound iron (NTBI) was evaluated as an index of ironoverload in a cross-sectional study in 74 non-transfused patients with thalassaemia intermedia (TI). Mean NTBI (2·92 ± 3·43 μmol/l), serumferritin (1023 ± 780 ng/ml) and liver iron concentration (LIC; 9·0 ± 7·4 mg Fe/g dry weight) were increased above reference-range levels. Significant positive correlations occurred between mean NTBI and LIC (Pearson correlation 0·36; $P = 0·002$) and serum ferritin (Pearson correlation 0·421; $P < 0·0001$); with higher levels observed in splenectomised patients. NTBI assessment has potential as a simple reliable approach to determining iron status in TI.

Keywords: iron overload, non-transferrin-bound iron, thalassaemia intermedia.

Anaemia in patients with thalassaemia intermedia (TI) is typically mild and does not necessitate regular blood transfusion therapy until later in life. Nonetheless, patients remain prone to iron loading, primarily due to intestinal iron absorption and ineffective erythropoiesis (Origa *et al*, 2007; Taher *et al*, 2008), with the accumulation of approximately 2–5 g of iron per year, depending on the degree of bone marrow expansion and peripheral hemolysis (Origa *et al*, 2007). Patients with TI eventually develop complications of iron loading similar to those observed in thalassemia major (TM), including liver, heart and endocrine dysfunction (Origa *et al*, 2007; Taher *et al*, 2008).

The most commonly used methods for evaluating iron overload include measurement of serum ferritin and liver iron concentration (LIC). Assessment of serum ferritin levels is convenient, non-invasive and widely used (Pakbaz *et al*, 2007), but is likely to underestimate the severity of iron load in patients with TI (Origa *et al*, 2007; Pakbaz *et al*, 2007; Chirnomas *et al*, 2008; Taher *et al*, 2008). LIC and heart iron concentration assessed by R2 magnetic resonance imaging (MRI) are reliable approaches but their use is limited due to cost and the need for specialised equipment.

Non-transferrin-bound iron (NTBI) is a low-molecular-weight form of iron that is detected in conditions of iron overload when transferrin becomes fully saturated and is unable to bind excess iron. NTBI is thought to catalyse the formation of reactive radicals (Cighetti *et al*, 2002), and is known to cause direct oxidative damage (Cappellini *et al*, 2000). High levels of free intracellular iron are related to massive membrane damage and metabolic impairment in thalassaemia (Tavazzi *et al*, 2001). Several studies have demonstrated that NTBI is a good index of iron overload in TM (al-Refaie *et al*, 1992; Cabantchik *et al*, 2005); however, data in patients with TI are limited. The current study evaluated whether NTBI is a useful index of iron overload in non-transfused or minimally transfused patients with TI by assessing the relationship between NTBI and serum ferritin, LIC and disease-related parameters.

Materials and methods

This was a cross-sectional study of patients with TI treated at the Chronic Care Centre in Hazmieh, Lebanon. A simple random sample was obtained from 120 patients with TI aged ≥2 years. A total of 74 patients agreed to be included in the study and written informed consent was provided by all patients. Patient charts were reviewed and a medical history compiled, which included details of drug history, comorbid

illnesses and transfusional history. Direct determination of LIC was performed by R2 MRI using an established methodology (St Pierre *et al*, 2005). Blood samples were obtained for assessment of pretransfusion haemoglobin, steady-state serum ferritin levels and NTBI (stored at −20°C).

Serum NTBI content was assayed by high performance liquid chromatography (HPLC) according to the methods of Porter *et al* (1996), with minor modifications. Briefly, 450 µl of serum was added to 50 µl of nitrilotriacetic acid (NTA) 800 mmol/l (pH 7·0) and was allowed to stand for 20 min to remove iron (Fe) non-specifically bound to serum proteins and low-molecular-weight ligands by the excess of NTA; in principle, the scavenged NTBI was quantitatively converted to Fe–NTA complex. The solution was then ultrafiltered using an Amicon Centricon 30 microconcentrator (Amicon Corporation, Lexington, MA, USA), and the ultrafiltrate (20 µl), which contained the Fe–NTA complex, was injected directly into the HPLC system (PerkinElmer series 200 IC titanium pump module; PerkinElmer Life Science, Boston, MA, USA). Chromatographic conditions were as follows: flow rate, 1·5 ml/min; mobile phase, isocratic containing 20% acetonitrile and 3 mmol/l CP22 in 5 mmol/l sodium phosphate buffer, pH 7·0; visible detection, 450 nm. A standard curve was generated by injecting different concentrations of iron (from 0 to 100 µmol/l in steps of 10 µmol/l) prepared in a 100-fold excess of NTA. Standards were run routinely from 0 to 10 µmol/l (in 1 µmol/l steps). Under these conditions, the 0 µmol/l standard corresponded to 80 mmol/l of NTA.

In accordance with previous reports (Aruoma *et al*, 1988; Porter *et al*, 1996), all our normal controls had values of ≤0 (–0·72 ± 0·70 µmol/l) because transferrin captures iron from the Fe–NTA complex. Normal individuals always have negative NTBI values because samples are measured in parallel with a corresponding blank formed by water and NTA. Water *per se* contains small amounts of iron that are not bound by transferrin, whereas in samples, transferrin that is not completely saturated captures some iron from the Fe–NTA complex (Gosriwatana *et al*, 1999). Therefore, the blank subtraction makes the NTBI value in some samples negative.

Descriptive statistics are expressed as means ± standard deviation (SD) or percentages where appropriate. Bivariate correlations between study variables were performed using independent-samples *t*-test for categorical variables and Pearson correlation for continuous variables. A multivariate stepwise regression analysis was done to determine significant correlations where needed. All *P*-values are two sided with the level of significance set at <0·05.

Results and discussion

Data from 74 patients were included in this analysis (Table Ia). All patients were chelation naïve and none of the patients had evidence of hepatitis B or C infection. The mean NTBI, serum ferritin and LIC were above the reference-range levels in this chelation-naïve population, highlighting that many patients with TI will be at risk of significant iron-related morbidity and mortality; and reflecting the haematological heterogeneity of TI patients.

Among study variables, NTBI levels were only significantly correlated to splenectomy status and transfusion history (Table Ib). On multivariate analysis, only splenectomy

Table Ia. Patients' characteristics.

Parameter	Value
Number of patients (*n*)	74
Mean age ± SD, years (range)	26·5 ± 11·5 (8–54)
Male:female	33:41
Splenectomized, *n* (%)	59 (80)
Transfusion history, *n* (%)	
Naïve	20 (27)
Infrequent (few transfusions received in the past)	45 (61)
Regular (2–4 times/year)	9 (12)
Mean haemoglobin ± SD, g/l (range)	84·3 ± 18·6 (49–131)
Mean NTBI ± SD, µmol/l (range)	2·92 ± 3·43 (−3·71–8·5)
Mean serum ferritin ± SD, µg/l (range)	1023 ± 780 (29–3158)
Mean LIC ± SD, mg Fe/g dry weight (range)	9·0 ± 7·4 (0·5–32·1)

NTBI, non-transferrin-bound iron; LIC, liver iron concentration; SD, standard deviation.

Table Ib. Bivariate analysis showing correlations between study variables and iron overload parameters.

Variable	NTBI (µmol/l)	Ferritin (µg/l)	LIC (mg Fe/g dry weight)
*Categorical**			
Gender			
Male (*n* = 33)	2·24 ± 3·50	1027·2 ± 640·6	10·3 ± 8·4
Female (*n* = 41)	2·84 ± 3·41	879·0 ± 648·6	7·9 ± 6·4
P-value	0·868	0·329	0·169
Splenectomy			
Yes (*n* = 59)	4·10 ± 2·87	1116·7 ± 604·9	10·5 ± 6·8
No (*n* = 15)	−1·04 ± 1·65	369·7 ± 400·2	3·9 ± 7·4
P-value	**<0·001**	**0·001**	**<0·001**
Transfusion history			
Naïve (*n* = 20)	1·38 ± 3·84	567·8 ± 455·2	4·0 ± 3·3
Infrequent (*n* = 45)	3·10 ± 3·14	1184·0 ± 869·6	10·5 ± 7·8
Regular (*n* = 9)	5·41 ± 2·28	1116·7 ± 604·9	12·6 ± 7·0
P-value	**0·010**	**<0·001**	**0·001**
Continuous†			
Age (years)	0·117	0·367	0·350
P-value	0·323	**0·001**	**0·002**
Haemoglobin (g/l)	0·017	−0·187	−0·180
P-value	0·888	0·110	0·125

NTBI, non-transferrin-bound iron; LIC, liver iron concentration.
*Statistical correlation evaluated by independent-samples *t*-test; data presented as mean ± SD.
†Statistical correlation evaluated by Pearson Correlation; data presented as Pearson correlation co-efficient (*r*).

remained independently correlated with NTBI ($P < 0{\cdot}001$). Splenectomised patients had higher serum NTBI levels than non-splenectomised patients, which is consistent with previous observations in patients with TI (Fiorelli *et al*, 1990; Cappellini *et al*, 2000). This observation suggests that the intact spleen may be a reservoir of excess iron and may have a possible scavenging effect on iron free fractions including NTBI (Tavazzi *et al*, 2001). Although increasing age was associated with higher levels of serum ferritin and LIC, no direct correlation was observed with NTBI. Serum ferritin and LIC are a cumulative index of iron accumulation over time. By contrast, transferrin saturation and the increased NTBI of patients with saturated transferrin is an acute phenomenon. In TI, increased transferrin saturation is the consequence of ineffective erythropoiesis with an outpouring of catabolic iron at a rate which is 10–15 times normal. Thus, NTBI would emerge at an earlier age when total iron accumulation in the liver and serum ferritin are still at a relatively early stage. This is not the case in TM where blood transfusions almost completely suppress the ineffective erythropoiesis and free iron appears when transferrin is completely saturated and in presence of severe iron burden.

Nevertheless, there was a significant correlation between NTBI and LIC (Pearson correlation 0·36, $P = 0{\cdot}002$; Fig 1A) and NTBI and serum ferritin (Pearson correlation 0·421, $P < 0{\cdot}0001$; Fig 1B). This confirms previous findings in which NTBI has been shown to be a good index of iron overload (as assessed by serum ferritin and total serum iron) in regularly transfused TM patients (al-Refaie *et al*, 1992; Cabantchik *et al*, 2005). However, a recent study showed no correlation between NTBI and LIC or serum ferritin in patients with TM or TI (Piga *et al*, 2009). Regular chelation therapy in these patients may have modified the relationship of serum ferritin and LIC with NTBI (Piga *et al*, 2009). In contrast, patients enrolled in the current study were chelation naïve and had iron levels above the reference range. The large number of patients with high NTBI yet serum ferritin levels below 500 μg/l, signifies that free iron could be present with less total iron burden (low serum ferritin). This is again explained by the aforementioned pathophysiology of iron loading in TI and echoes studies showing that serum ferritin underestimates iron overload in TI patients (Taher *et al*, 2008). These observations have important implications for patient management, as assessment of serum ferritin alone may result in a delay in initiating chelation therapy and may, therefore, prolong patient exposure to high iron levels and the associated morbidity and mortality risks. In these patients we were unable to evaluate T2* to estimate myocardial iron accumulation. A recent study has shown that the presence of heart disease is associated with significantly higher NTBI values in patients with TI or TM, which supports the concept that increased severity of myocardial siderosis is caused by a critical expansion of the toxic chelatable iron pool (Piga *et al*, 2009).

In conclusion, this study demonstrated that in non-transfused or minimally transfused patients with TI, NTBI was detectable and above reference-range levels and was significantly correlated with LIC and serum ferritin in the overall population. Despite some patients having low serum ferritin levels, NTBI was increased, therefore suggesting that the assessment of NTBI levels has potential as a simple and reliable approach to determine the iron status of patients with TI.

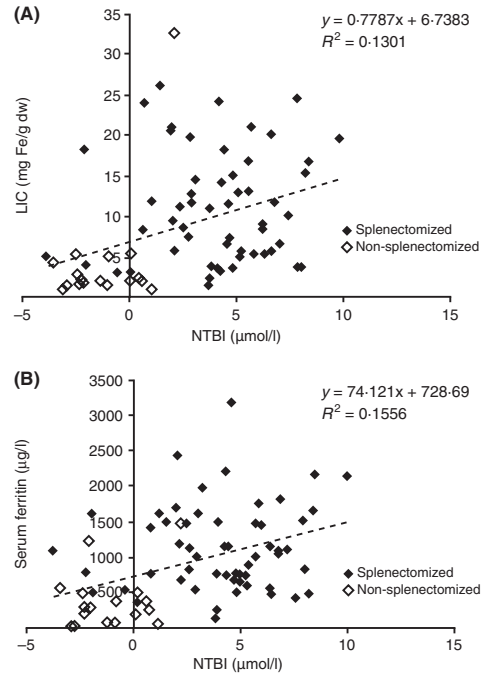

Fig 1. Correlation between non-transferrin-bound iron (NTBI) and (A) liver iron concentration (LIC) and (B) serum ferritin.

Conflict of interest disclosures

Dr Taher has received research grants and lecture fees from Novartis pharmaceuticals. Dr Musallam has no relevant conflicts of interest to disclose. Dr El Rassi has no relevant conflicts of interest to disclose. Dr Duca has no relevant conflicts of interest to disclose. Dr Inati has received lecture fees from Novartis Pharmaceuticals. Dr Koussa has no relevant conflicts of interest to disclose. Dr Cappellini has received lecture fees from Novartis Pharmaceuticals.

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