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**Original Article**

# Detection of Hepatotoxicity by Non-Transferrin Bound Iron in Beta Thalassemia Major

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**Background:** Hepatotoxicity due to iron overload as a result of multiple transfusions along with concomitant hepatotropic infections has not been studied in detail.
**Objective:** The objective of our study was to estimate the relationship between the extent of hepatocellular injury as reflected by serum levels of alanine aminotransferase (ALT) and iron status indices like Ferritin and Non-transferrin bound iron(NTBI) .
**Materials & Methods:** A total of 137 transfusion dependent thalassemic patients with median age of 10 (range 3-21) years (73 males; 58 females) participated in the study. Total 76 (58%) patients were positive for hepatitis B&C and 55 (42%) negative. Serum ALT and Aspartate aminotransferase (AST) were more significantly raised in hepatitis positive patients in comparison to other thalassemics. Significant correlation was found between hepatocellular damage (raised ALT) and NTBI (r=0.305) in thalassemic patients (figure. 3). No correlation was seen between serum ferritin and hepatocellular injury (r=0.028). We compared ROC curves of NTBI and Ferritin to detect hepatotoxicity (table 2). Serum NTBI proved to be better between the two. ROC curve of serum NTBI is shown (figure 4). Using cutoff value of 1.75uM and with AUC of 0.81, serum NTBI can detect hepatocellular damage in beta thalassemia major (BTM) with 76%sensitivity and 88% specificity.
**Conclusion**: A high percentage of thalassemic patients (58%) suffer from viral hepatitis after getting transfused. NTBI is the best measure of iron toxicity showing the good association with iron overload complications as compared to serum ferritin.
**Key words:** NTBI, Ferritin, Thalassemia major

## Introduction

Hepatic injury due to iron overload has been emerged as a main cause of morbidity in patients with ß-thalassemia major.[1] In addition, these patients are also infected with Hepatitis B and C particularly in the developing countries where blood screening facilities are not sufficient.[2] Hepatotropic infections or hepatic siderosis both factors may act either synergistically or independently in promoting chronic liver disease.[3]

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In spite of high prevalence of hepatitis C infection; thalassemia- associated liver damage has been insufficiently characterized in our patients and requires consideration.

Measurement of Liver iron concentration (LIC) in biopsy specimen is a gold standard for the assessment of total iron burden in thalassemic patients but its invasiveness has restricted its acceptability to patients.[4]

According to the latest international practice guidelines, measurement of serum ferritin is still the first choice tool for monitoring iron overload in thalassemic patients in the absence of confounding factors.[5] Ferritin is an acute phase reactant protein and its levels are falsely raised in viral hepatitis.[6] Hence, does not reflect the true picture

of iron overload induced hepatotoxicity. In iron overload conditions such as thalassemia major, excess iron saturates the binding capacity of transferrin and appears in circulation as insoluble form known as non-transferrin bound iron (NTBI). NTBI is capable of freely permeating into the organ resulting in end organs failure.[7] NTBI may be more relevant biomarker while looking for an association with iron overload induced hepatic damage.

## Methods

It was a cross-sectional study-carried out at Department of Pathology of National University of Sciences & Technology (NUST) in collaboration with thalassemia center Rawalpindi Pakistan. The study protocol was approved by institutional review and ethical committees of NUST Pakistan.

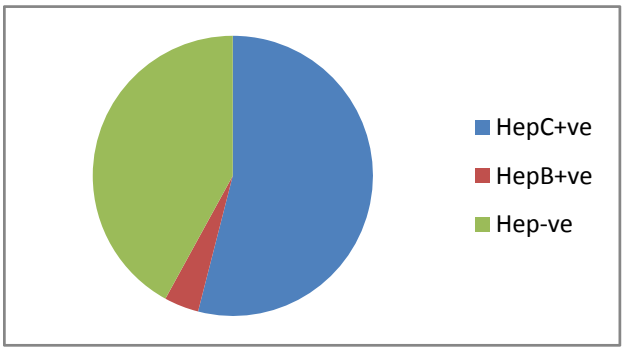
A total of 137 transfusion dependent thalassemic patients were randomly enrolled from thalassemia centre, Rawalpindi registry after informed consent. Four of them were found unfit for the study according to our exclusion criteria (congenital heart disease, autoimmune disease, metabolic disorder). The medical history including age of diagnosis, transfusion and chelation therapy were recorded. Liver function tests were assessed on Chemistry autoanalyzer (Hitachi). Serum ferritin was measured on ACCESS II reagent Pack (USA). Serum NTBI was measured on Analytikagena following a method described by Jittangprasert et al.[8] 450µL of serum sample was taken. 50µL of 0.2M NTA (pH: 7.0) was added. The mixture was allowed to stand for 30min. at room temperature. The solution was then ultrafiltered using an Amicon microcon YM-30filter(MW 30,000 cut-off Millipore Corporation, Bedford, MA, USA) with an applied centrifugal force of 5,000 rpm for 60 minutes to separate Fe(III) NTA complex from transferrin. Standard iron solutions at various concentrations (0.15-20µM) were prepared in 0.02M NTA (pH 7.0) and calibration curve was taken on Analytikagena (Spectrophotometer). An aliquot of 400 µL of serum ultrafiltrate was diluted 1:2(v/v) with 0.50M. HEPES buffer (pH 7.0). 100 µL of reducing agent 0.05M Thioglycolic acid (TGA) was added. 100 µL of 0.05M Bathophenanthroline disulfonic acid disodium salt (BPT), a chromogen for iron (II) was added in each of the tube. The solution was then equilibrated for 90min at room temperature in order for the formation of colored complex to reach equilibrium before measurement of absorbance at 537.0 nm. All the samples were incubated at room temperature for 90min for the color production. The absorbance of the samples was taken at 537nm on spectrophotometer. CV of the assay was less than 5 %.

### Statistical analysis

Statistical analysis was performed using SPSS 16 (SPSS Inc, Chicago) and MedCalc software version 10. Diagnostic accuracy of NTBI was assessed using receiver operating characteristics (ROC) curve analysis. We set the cut-off value at which the discrimination between the cases with positive and negative diagnosis was optimal. The associations between NTBI and ALT were quantified with 95% confidence intervals (95% CI). Correlation between NTBI and ALT was tested using Spearman's correlation test. A two tailed p value of <0.05 was taken as significant.

## Results

131 thalassemic children with median age of 10 (range 3-21) years (73 males; 58 females) participated in the study. The pre transfusion hemoglobin and serum ferritin were 8.7(3.2-14.8) g/dL and 2478(792-15334) µg/L respectively. Total 76 (58%) patients were positive for hepatitis B&C and 55 (42%) negative (figure 1). Serum ALT, AST, Bilirubin and albumin were also found raised in 25% patients other than hepatitis positive. However serum ALT and AST were more significantly raised in positive patients in comparison to other thalassemics (table 1).




Hep C += 54%, Hep B + = 4%%, Hep –ve = 42%

**Figure 1: Percentages of hepatitis positive and hepatitis negative Pakistani BTM patients**

**Table 1: Difference of hepatic profile between hepatitis negative and hepatitis positive thalassemia major patients (n=131)**

| Parameters | Hepatitis positive Median(IQ) n=60 | Hepatitis negative Median(IQ) n=71 | P value |
|---|---|---|---|
| **Ferritin(µg/L)** | 4349(2782-5927) | 3338(2189-5506) | 0.02 |
| **ALT(U/L)** | 143(87-187) | 116(56-160) | 0.03 |
| **AST(U/L)** | 67(51-110) | 65(50-94) | NS(.775) |
| **ALP(U/L)** | 450(390-507) | 408(319-504) | 0.05 |
| **Bilurubin(umol/L)** | 20(16-24) | 17(14-19) | 0.00 |
| **Albumin(g/L)** | 40(41-44) | 44(38-42) | 659(NS) |

It suggests increased hepatocellular injury induced by transfused iron as well as viral hepatitis. Serum ALT activity was generally higher than serum AST activity. A positive correlation existed between serum NTBI and Transferrin saturation(r=0.45) (figure 2). Using cut-off values of transferrin saturation 70% and NTBI above or below 0, almost all the patients with elevated ALT activity were confined to the group that was NTBI positive with transferrin saturation above 70% (figure 2).

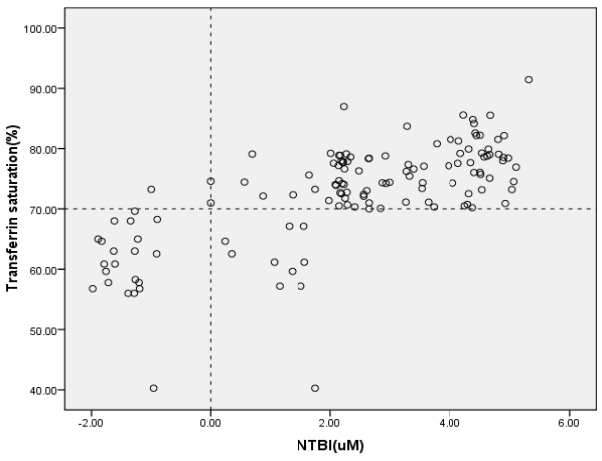
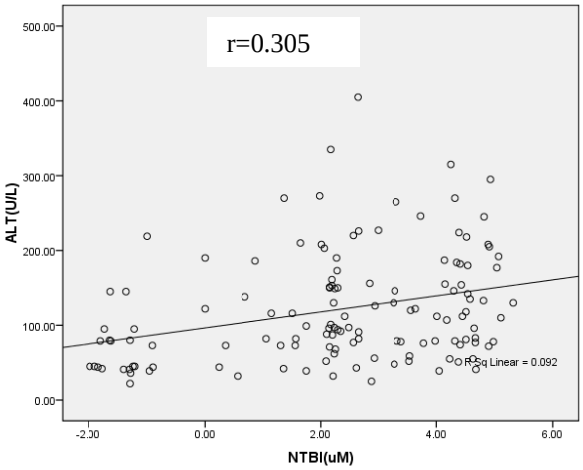
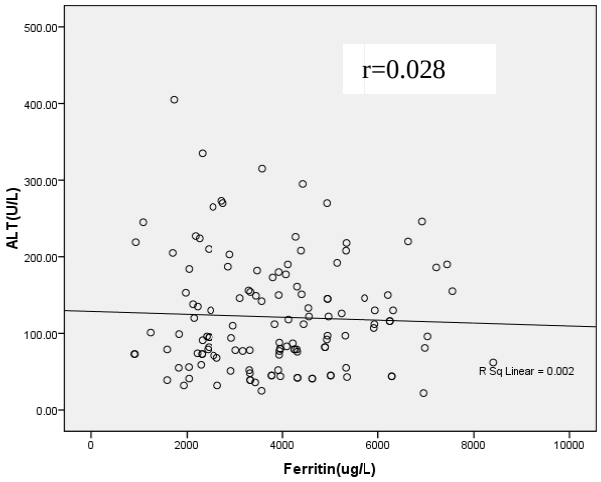



**Figure 2: Correlation between NTBI and transferrin saturation in BTM patients with hepatotoxicity**

Both serum ferritin and NTBI were evaluated in correlation with hepatic complications in thalassemic patients. Significant correlation was found between hepatocellular damage (raised ALT) and NTBI (r=0.305) (figure 3).




**3A**




**3B:**

**Figure 3: Comparison of Correlation of NTBI (A) and Ferritin (B) with ALT in BTM patient**

No correlation was seen between serum ferritin and hepatocellular injury (r=0.028) shown in figure 3. We compared ROC curves of NTBI and Ferritin to detect hepatotoxicity (Table 2). Serum NTBI proved to be the best amongst all others. ROC curve of serum NTBI is shown (figure. 4). Using cutoff value of 1.75uM and with AUC of 0.81, serum NTBI can detect hepatocellular dam-

age in BTM with 76%sensitivity and 88% specificity (figure 4).

**Table 2: Comparison of ROC curve analysis among different iron overload parameters for hepatic complications in hepatitis negative BTM patients (n=131)**

| ROC curve analysis | NTBI | Ferritin |
|---|---|---|
| AUC(95%CI) | 0.815**(0.70-0.89) | 0.615(0.49-0.72) |
| Cut-off value | 1.75 | 2772 |
| Sensitivity | 76 | 55.60 |
| Specificity | 88.2 | 70.6 |
| +LR | 6.45 | 1.89 |
| -LR | 0.27 | 0.63 |

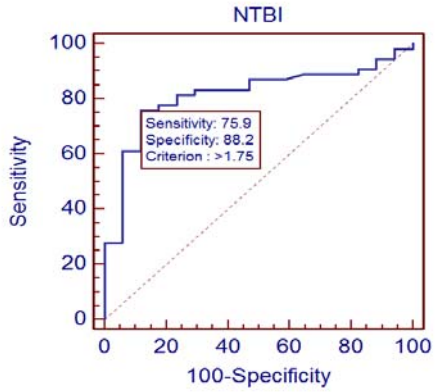



**Figure 4: Receiver Operating Characteristics (ROC) curve of NTBI to determine Hepatic complications (elevated ALT&AST) in BTM patients**

## Discussion

Hepatic complications in thalassemia major patients are both due to iron overload and viral hepatitis.[4] Although, early and regular blood transfusion in β-thalassemia major patients reduces the complications and prolongs survival. However, if there is a violation of "safe blood transfusion", these patients are diverted to new clinical challenges, especially hepatitis B & C viral infection.[9] Hepatitis B virus infection has been controlled to a great extent by the invention of vaccine against this virus. HCV infection is gaining attention particularly as one of the major complications in multiply transfused patients during the last decade.[10] Our study also indicated high percentage of patients suffering from viral hepatitis (58%) which is distressing. The secondary iron overload due to multiple transfusions is equally responsible for hepatic dysfunction in thalassemia major. We observed elevated serum ALT activity in large number of patients (25%) other than those seropositive for hepatitis B&C (Table 1). Therefore, we need to address both problems simultaneously to prevent hepatic dysfunction in thalassemia major. For practical reasons serum ferritin is the most commonly used as the most reliable marker of iron overload measurement in thalassemic patients.[11] The cutoff point of serum ferritin for estimating iron toxicity level differs in studies from 1000 µg/L to 3000 µg/L.[12-13] This signifies the need of NTBI as a toxicity measure of iron overload in thalassemia major. As only free iron participates in tissue damage so, serum ferritin can not identify any harmful effects of iron, unless the measure of bound iron is also a marker of free iron. Our study also observed the significant positive correlation of NTBI with hepatic damage (raised ALT) rather than serum ferritin. Bearing in mind this observation we describe that serum ferritin may increase to meet oxidative stress when it is induced by NTBI and a raised level of bound iron may initially reflect increased stored body iron. However, in those conditions where such measure does not reflect free iron, the level of stored body iron may not be a suitable spotlight for determining harmful effects of iron. These factors explain why there have been inconsistent results between serum markers of stored body iron and complications in different studies on thalassemic patients. This study is applicable to all thalassemic patients irrespective of age and sex.

## Limitations

As chelation therapy removes the free iron from the body. We could not measure the levels of NTBI in correlation neither with Desferrioxamine nor Deferiprone because of patient's non-compliance.

## Conclusion

Hepatic dysfunction is a frequent complication among Pakistani BTM patients both due to iron overload and viral hepatitis (58 %). NTBI is a better measure of iron toxicity showing good association with iron overload complications as compared to serum ferritin.

## Recommendations

Serious measures are required in our set up to ensure a safe blood transfusion, so as to cut down the prevalence of HCV in multiply transfused thalassemic patients. Uniform strict criteria for donor selection should be adopted in all thalassemia centers. NTBI appears to be more significant variable than serum ferritin that deserves consideration in evaluating the risks and designing optimal treatment for transfusion-dependent thalassemic patients.

### Disclosures

None

## Acknowledgement

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