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Title Page
Clinical and methodological factors affecting non-transferrin-bound iron (NTBI) values using a novel fluorescent bead assay.

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Abbreviations:
AAS, atomic absorption standard; AlAT, alanine-aminotransferase; CFB, control fluorescent beads; CI, confidence interval; CIC, cardiac iron content; CSA, congenital sideroblastic anaemia; DBA, Diamodn-Blackfan anaemia; DCI, directly chelatable iron; DFO, deferoxamine; DFP, deferiprone; DFX, deferasirox; ELISA, enzyme-linked immunosorbent assay; f.c., final concentration; FBC, full blood count; IQR inter-quartile range; LIC, liver iron content; LPI, labile plasma iron; MDS, Myelodysplastic syndrome; MOPS, 3-(N-morpholino)propanesulfonic acid; NRBC, nucleated red blood cells; NTA, nitrilotriacetic acid; NTBI, non-transferrin-bound iron; SD standard deviation; SF, serum ferritin; sTfR, soluble transferrin receptors; Tf, transferrin; TfSat, transferrin saturation; ULN, upper limit of normal; UV, ultraviolet;

Abstract

Non-transferrin-bound iron (NTBI) is a heterogeneously speciated plasma iron, typically detectable when transferrin saturation (TfSat) exceeds 75%. Here we examine factors affecting NTBI levels by a recently discovered direct chelator-based (CP851) fluorescent bead-linked flow-cytometric assay (bead-NTBI), compared with the established indirect nitrilotriacetate (NTA) assay in 122 iron-overloaded patients, including 64 on recent iron chelation therapy and 13 healthy volunteers. Both methods correlated (r=0.57, p<0.0001) but with low agreement, attributable to two major factors: (1) the NTA method, unlike the bead method, is highly dependent on TfSat, with NTBI under-estimation at low TfSat and over-estimation once Tf is saturated, (2) the bead method detects <3-fold higher values than the NTA assay in patients on recent deferiprone-containing chelation due to greater detection of chelate complexes but lower values for patients on deferasirox. The optimal timing of sample collection relative to chelation dosing requires further study. Patients with splenectomy, high storage iron and increased erythropoiesis had greater discrepancy between assays, consistent with differential access by both methods to the NTBI pools associated with these clinical variables. The bead-NTBI assay has advantages over the NTA assay, being less dependent on TfSat, hence of less tendency for false-negative or false-positive values at low and high TfSat, respectively.

Keywords: iron, iron overload, iron biochemistry, thalassaemia, erythropoiesis, NTBI, assay

Introduction

Plasma non-transferrin-bound iron (NTBI), first described in 1978[1], is a pathological iron pool detectable when Tf saturation exceeds 75%[2–4]. NTBI appears when iron influx into the plasma compartment exceeds iron efflux, e.g. with iron overload, ineffective erythropoiesis, or decreased transferrin iron clearance in erythroid hypoplasia[5]. NTBI is considered the main conduit of hepatic[6–8] and extra-hepatic[9–12] iron loading of tissues, under haemosiderotic conditions. Quantitating NTBI is of value in understanding NTBI generation under different pathophysiological settings[5] but can also be potentially useful in the management of iron-overloaded patients. However using established methods for NTBI quantitation, clear consensus and guidelines on how to use NTBI measurement in patient management have yet to emerge. This is partly because NTBI is multi-speciated, consisting of a range of iron-citrate[13,14], albumin-bound complexes[13], glycated protein-iron complexes[15,16] or iron-chelate complexes in recently chelated patients[17]. Consequently it is unlikely that NTBI assays relying on different principles will measure different NTBI species to the same extent. Hence a consistent pattern of association between NTBI values and clinical outcomes has yet to emerge.

There is therefore a need to identify a robust and well-characterised NTBI assay that can be applied in a standardised manner in the management of iron–overloaded patients. A range of NTBI methods used previously differ considerably in their detection principles and in reported reference ranges[18]. The most long-standing and frequently reported NTBI method[19] involves iron capture from NTBI by a high concentration of a low affinity/specificity iron chelator, nitrilotriacetic acid (NTA, 80mM), followed by ultrafiltration and detection of NTA-iron by HPLC[2,19] or spectrophotometrically[3]. Another approach is measuring NTBI indirectly by quantifying the redox-active subset of NTBI, which has been termed the 'labile plasma iron' (LPI) assay[20]. A further approach is measuring the directly chelatable iron (DCI) with a fluorophore-labelled high-affinity chelator[18,21,22] but background fluorescence in plasma may interfere with data interpretation. Most recently, an adaptation of this approach was described, using a high-affinity fluorescent chelator CP851, covalently linked to magnetic beads with fluorescence signal separated flow-cytometrically from plasma autofluorescence[23]. This potentially circumvents the auto-fluorescence problem in the plasma sample and problems related to indirect capture of NTBI by NTA.

The initial paper describing the bead method[23] examined only 30 patients and did not therefore explore the variables affecting the agreement between the NTA and the bead method systematically. In particular, the effects of TfSat, chelators, splenectomy status and underlying diagnosis were not explored. A recent round robin[18] comparing various NTBI and

LPI methods on 60 patients, reported their overall lack of agreement in absolute values despite similar correlations, but did not specifically look at the agreement between these two methods and could not therefore comment on the possible reasons for their poor agreement. Here, we compare levels of NTBI detected by this assay with the NTA method in various clinical conditions, including 122 iron-overloaded patients with approximately half (n=64) receiving regular chelation therapy. Part of this work was presented as Abstract no. 241 at BioIron Conference Sep 6-10, 2015 in Zhejiang University, China.

Materials and Methods

Patients

122 clinical blood samples from iron-overloaded patients and 13 healthy volunteers, obtained from 3 hospitals (affiliations 2, 4 and 5), were analysed using the CP851-NTBI assay and NTA-NTBI assay. Diagnoses included are listed in **Table I**. Ethical approval was obtained for the study at the respective institutions where blood samples were collected and patients signed informed consent forms before sample collection.

NTBI assays

NTA-NTBI assay

The NTA-NTBI method previously described[19] was adopted with minor modifications. Briefly, 0.02mL of 800mM NTA (at pH=7) was added to 0.18mL serum and allowed to stand for 30min at 22°C. The solution was ultrafiltered using Whatman Vectaspin ultracentrifugation devices (30 kDa) at 12320g and the ultrafiltrate (0.02mL) injected directly onto an HPLC column (ChromSpher-ODS, 5μM, 100x3mm, glass column fitted with an appropriate guard column) equilibrated with 5% acetonitrile and 3mM DFP in 5mM MOPS (pH=7.8). The NTA-iron complex then exchanges to form the DFP–iron complex detected at 460nm by a Waters 996 PDA. Injecting standard concentrations of iron prepared in 80mM NTA generated a standard curve. The 800mM NTA solution used to treat the samples and prepare the standards is treated with 2μM iron to normalize the background iron that contaminates reagents. This means that the zero standard gives a positive signal since it contains the added background iron as an NTA–complex. When unsaturated transferrin is present in sera, this additional background iron can be donated to vacant transferrin sites resulting in a loss of the background signal and yielding a negative NTBI value.

CP851 bead-NTBI assay

The standards for this assay were prepared as follows: 1mM iron-NTA complex (1:2.5 molar ratio), prepared from 100mM NTA and 18mM atomic absorption standard iron solution, was

diluted with MilliQ water to a final concentration (f.c.) between 0-100µM. For the standard curve, 120µL quantities of probe-labelled bead suspensions were incubated with 20µL of buffered NTA-iron solutions of known concentration for 20min at room temperature, with subsequent addition of 20µL normal control serum (without free iron) and 40µL paraformaldehyde (10% in MOPS) at 2% f.c. The suspensions in sealed 96-well plates were incubated at 37°C for 16h with shaking before fluorescence measurement by flow-cytometry. For serum samples of unknown iron concentrations, 140µL quantities of beads were incubated with 20µL of serum samples for 20min, with subsequent addition of 40µL paraformaldehyde at 2% (f.c.). In this study, we used chelatable fluorescent beads (CFB) with normal human mixed serum as control to set up the fluorescence at 100% and the relative fluorescence of CFB with patient serum was calculated accordingly. Measurements were carried out on Beckman Coulter FC500 flow-cytometer and analysis on Cell-Quest and FlowJo software. Gates were based on dot-plots of untreated bead populations. Median fluorescence of 10,000 events was recorded and corrected for bead auto-fluorescence. Standard curve was fitted with variable-slope sigmoidal dose-response function.

Transferrin Saturation
TfSat was determined by the urea-gel method[24] with band quantitation using Scion Image software, normal reference range 16-56% (mean 36%).

Routine blood test results and standard of medical care monitoring
Haematology tests: FBC and red cell indices, reticulocytes, NRBC, serum ALT, bilirubin, and ferritin were performed routinely in hospital laboratories, sTfR was measured using ELISA (R&D Systems).

Liver iron content (LIC) was obtained from liver T2*[25] or liver R2 Ferriscan[26], and cardiac iron content (CIC) obtained from cardiac T2*[27]. Medical records review provided information about chelation therapy, transfusion, and splenectomy status.

Statistics
The data was presented descriptively using mean±standard deviation (SD) or median± interquartile range (IQR) where appropriate, differences between subgroups were calculated using Wilcoxon test or paired t-test, dependent on distribution assumptions. 95% confidence interval (CI) follows slope and Spearman or Pearson correlation coefficient value in brackets. Graphpad Prism Ver. 6.0 plots were used for slope comparison and Bland-Altman plots[28] to illustrate agreement between methods. A $p < 0.05$ was deemed statistically significant.

Results

Correlations, distributions and agreement between NTA-NTBI and bead-NTBI were first examined. The TfSat effects on measured values were compared, followed by the effects of chelation therapy, erythropoiesis, storage iron and splenectomy status on NTBI measured by both methods.

Correlation and agreement of the two assays in all samples

There was a medium-strong correlation between bead-NTBI and NTA-NTBI for all samples (**Fig 1A**). Because negative values were obtained in the NTA assay, the graph was re-plotted treating all negative values as zero (**Fig 1C**). In Bland-Altman analysis (**Figs 1B,1D**), the differences between methods notably increased at mean NTBI>2.5μM being even greater above mean values of 4μM. For mean values between <1 and 4μM the bias is not constant, being negative at <0μM (NTA method less than bead method) and positive between 0 and 2μM (bead higher than NTA). Treating negative values as zero does not improve agreement (**Fig 1D**), suggesting that negative values are not the main reason for lack of agreement.

The distribution of NTBI values by the two methods

The NTBI distributions for both methods differ considerably between the assays (**Fig 2A**). With the NTA-NTBI (black) there is a clear population of negative values that is absent with the bead-NTBI (red). There is then a second population of positive NTA values (mode=1.5μM) that is absent with the bead-NTBI where the mode=0μM. Finally there is a long positive tail of high bead-NTBI up to 14μM that is absent with the NTA method. Further analysis below aims to determine the meaning of these distributional differences.

Relationship of TfSat to NTBI values by the two methods

NTBI values are related to TfSat by NTA assay but not bead assay. The relationship of NTBI values by both methods to TfSat is presented in the **inset of Fig 2A**. TfSat has a strong relationship to the NTA-NTBI over its negative range, but not to the bead-NTBI. In particular, the NTA-NTBI values fall with decreasing TfSat, unlike with the bead assay, with the NTA-NTBI becoming negative for TfSat<80%, but no such effect is seen with the bead method, which confirms previously published results on a smaller group of patients[18]. Because of the relationship of NTBI to TfSat by the NTA method and not the bead method, we examined the distribution of TfSat shown in **Fig 2B** for the bead-NTBI (left) and NTA-NTBI (right) for samples where positive (red) or negative (black) NTBI values were obtained. It appears that whereas with the bead-NTBI TfSat has a similar (p=0.84) distribution for patients with positive or negative NTBI values, by contrast the negative

NTA-NTBI are largely confined to patients with TfSat<60% and the positive values to patients with TfSat>80% (p<0.0001). This supports the relevance of low TfSat to negative NTA-NTBI but not bead-NTBI values.

Relevance of high TfSat to false-positive values with NTA assay. 'True-negative' bead-NTBI values (below ULN=0.68µM i.e. 3xSD above normal control mean=−0.1µM, SD=0.26µM, marked as dashed line in **Fig 1A**) were plotted against NTA-NTBI with the point size reflecting TfSat (**Fig 2D**, note Figures 2C,D are derived from Figure 1A). Positive NTA-NTBI associates *exclusively* with high TfSat while negative NTA-NTBI almost *exclusively* with low TfSat (93±11.3% vs 41.5±18.2%, p<0.0001). This means that the positive values by the NTA method that are negative by the bead method associate with high TfSat and are 'false-positive'. This is consistent with iron being stripped off holotransferrin by NTA and not by CP851 (discussed). In contrast, no such distributional TfSat differences were seen between the negative and positive bead-NTBI values (on both sides of the red dotted line, **Figs 2B,D**). It is also possible that for other NTA-NTBI assays (N3 in the round robin[18], or using Co or Mn blocking[29]) the relationship with the TfSat may be different.

Relevance of low TfSat to false-negative values with NTA assay. The NTA-NTBI values were plotted against 'true-positive' bead-NTBI values (above ULN, **Fig 2C,** mark the range change on the x-axis in **Fig 2C vs 2D**) with the point size reflecting TfSat. It appears that the 'true-positive' bead-NTBI is also typically positive by the NTA method only when associated with high TfSat. In the small number of values where the NTA-NTBI is negative, TfSat is low. Thus 'false-negative' NTA-NTBI values occur only in the presence of apotransferrin.

Relationship of chelation therapy and NTBI values by both methods

The presence of iron-chelate complexes or iron-free chelator, could in principle influence the assay behaviour *in vitro* [17,23,30]. We examined these potential effects by comparing values in regularly chelated patients with those not receiving chelation. Both NTBI assays are plotted for chelated and un-chelated patients in **Fig 3A**. The range of values differs in the upper (no chelation) and lower panels (recent chelation), particularly for the bead-NTBI (red), where there is a 'tail' of high values (5-15µM) in chelated patients (**Figs 3B,D**). In principle, this could represent iron-chelate complexes detected as bead-NTBI. With the NTA-NTBI (black), the difference between chelated and un-chelated patients is subtler, but it appears that the proportion of patients with negative values decreases while that of patients with slightly positive values (up to 2µM) increases on chelation (**Figs 3B,E**). This could again represent the effects of iron-chelate complexes on the assays.

In order to investigate this in more detail, the values obtained with both assays were plotted for patients on different chelation regimens **(Figs 3F,G,H,I)**. Notably, values obtained on the

same samples with the bead assay (max. 15µM) are about 3-fold higher than with the NTA assay (max. 5µM) for deferiprone or combination (desferrioxamine+deferiprone) treated patients but not for deferasirox, where bead-NTBI values are actually *lower* than NTA-NTBI. Patients receiving desferrioxamine usually show about 2-3-fold higher values with the bead assay than the NTA-NTBI. NTBI levels correlated within the desferrioxamine (0.47 (0.01, 0.77), p=0.05), and the deferiprone (0.62, (0.34, 0.81) p=0.0003) groups, but without agreement of absolute values, while in the deferasirox patients correlation was absent.

Differences in patient sampling on deferasirox and deferiprone offer partial explanation. All patients receiving deferasirox had a 72h chelation washout, strictly observed before sampling, making the presence of the contaminating iron-deferasirox complexes unlikely. By contrast, in most of the deferiprone-treated patient samples, where 72h washout was not observed, the iron-chelate complexes would be expected to be present[17,23]. It appears that the complexes of deferiprone are more readily detectable as bead-NTBI than NTA-NTBI but different incubation times within the assay procedure are probably partly responsible for it. The detection of high levels by the bead assay in 2 patients on desferrioxamine (**Fig 3G**) is not predicted from in vitro evaluation of the bead assay[23] and is difficult to explain unless patients were taking deferiprone.

Other factors affecting relationships between assays

Using univariate analysis, we investigated the effects of diagnoses, transfusion status, erythropoiesis (by sTfR), splenectomy, and storage iron, on the levels of NTBI by both assays. There was no relationship between NTBI and diagnoses, but both NTBI methods were differentially affected by high erythropoietic status, splenectomy and high iron storage (as detailed in Supplementary Material: Subgroup univariate analysis, **Table S3, Figs S4, S5, S6, S7**).

Multiple regression

Multiple linear regression models were built to test which predictors explain differences between the NTA-NTBI and the bead-NTBI. The same or additional predictors may resurface as relevant when the absolute difference (bias) between methods is modelled and this was attempted as a control analysis. Furthermore, we have modelled transferrin saturation using the same set of potential predictors as for the other models (**Table II**). The bead-NTBI model explained 25% of the variability in NTBI using *TfSat*, *splenectomy*, and *DFO+DFP* as positive predictors. The NTA-NTBI model explained >75% of the variability in NTBI, with *TfSat*, *splenectomy* as positive, and *sTfR* as negative predictors. The bias model, i.e. the difference CP851-NTBI less NTA-NTBI, was predicted negatively by *TfSat* and *DFX*, and positively by *splenectomy*, *DFO+DFP*, *chelation* (yes/no), and "*normal*", in a model explaining 24% of the NTBI variability. The TfSat model explained 42% of the NTBI

variability using *thalassaemia*, *Eβ-thalassaemia*, *MDS*, *CSA*, *DBA*, and *SF* as positive predictors.

Discussion

This study examined a range of iron-overloaded patients with and without iron chelation, which allowed comparison of the established NTA-based NTBI assay (NTA-NTBI) with a novel fluorescent bead-based assay (bead-NTBI) under a wide range of clinical conditions. Overall, while significant correlations exist between both methods, absolute values differ with wide 95% limits of agreement, consistent with the recent round robin of NTBI assays[18]. Here, by comparing values in a large number of iron-overloaded patients, we examine how both assays are differently affected by identifiable variables. These are relevant to the application and interpretation of both NTBI assays in specific patient populations. Two systematic differences between the assays have been identified. Firstly, the NTA-NTBI is highly affected by TfSat, leading to under-estimations at low TfSat and over-estimation once transferrin is saturated. By contrast, the bead-NTBI is less dependent on either high or low TfSat. Secondly, while both assays give increased NTBI values in recently deferiprone-treated patients, due to detection of chelate-iron complexes, this effect is more pronounced with the bead-NTBI, leading to a further lack of agreement between the two assays.

Inspection of the NTBI distribution histograms for both assays, combined with the knowledge of the contrasting chelating properties of NTA and the hexadentate CP851 used in the bead method, provides insight into why agreement is not high. With the NTA-NTBI, two major peaks are seen, first centring on -2.5μM, and the second peak on 1.5μM skewed rightwards. With the bead-NTBI these peaks are essentially absent with most low values clustered around zero and with a much more pronounced right skew for positive NTBI. These differences are consistent with the known properties of the 'capture' mechanisms of the assay chelators. Negative NTBI values obtained with the NTA method have been attributed to shuttling of iron present in 80mM NTA onto apotransferrin during the initial incubation[2,31,32]. This shuttling iron donation effect, due the greater stability of the hexadentate bead chelators, is absent in the bead method[23]. 80mM NTA not only donates chelated iron to iron-binding apotransferrin sites, but also removes iron from holotransferrin in a time- and concentration-dependent manner[3], so that at 30 minutes 80mM NTA mobilised 1-2% of transferrin iron (physiological concentration, TfSat=50%). This represents 0.35-0.7μM transferrin-bound iron potentially detected as 'NTBI' but could be as much as 1.4μM with TfSat=100% in our patients. Others noted similar effects[33,34].

The impact of such high iron removal from transferrin has not been previously characterised and here the comparison with the bead method, where iron is not stripped from holotransferrin (<0.2%) by the hexadentate chelator[23], demonstrates this effect more clearly. Given the robustness towards transferrin of the bead-assay, it can be used as a reference in an attempt to examine transferrin dependence of the NTA-method. Closer inspection of the normal and pathological bead-NTBI distributions suggests a cut-off above which values can be regarded as 'true-positive' and provides supportive evidence for the mechanisms underlying 'false-positive' and 'false-negative' values obtained with the NTA assay. With the bead-NTBI, values centring in the narrow dominant peak around the mode -0.04μM (**Fig 2A**) are likely to represent an absence of true NTBI, given the comparable spread around the mean of the normal serum samples (-0.1±0.26μM with ULN of 0.68μM mean+3SD, SD=0.26μM, **Figs 2A,S5K**). Bead-NTBI values >0.68μM are therefore likely to represent 'true-positive' NTBI. Clearly 'false-negative' values by the NTA method would then be those negative values obtained when the bead assay gives positive values >0.68μM. When this was checked, all the 'false-negative' NTA values occurred in samples with non-saturated transferrin (45, 18, 48%, **Fig 2C**) where the predicted percentage of apotransferrin, that can act as acceptor for iron shuttled by NTA, approaches 30%, 65% and 28% respectively[35]. By contrast, 'false-positive' NTA-NTBI would be those positive values that correspond to bead-NTBI<0.68μM. All such 'false-positive' NTA values had highly saturated transferrin (mean 93%, range 62-100%) supporting the concept that such 'false-positive' NTBI values are obtained from the scavenging of iron from highly saturated transferrin.

The second factor, contributing to differences between both assays, is the effect of iron chelators or their iron complexes. With the NTA-NTBI, iron-free chelators present in plasma can act as acceptors for NTA-bound iron, potentially leading to the NTBI underestimation[31,32]. This donation can be blocked in the NTA assay by adding an excess of aluminium to samples before processing [30,32]. This is not a problem for the bead-NTBI, as the greater stability of the CP851-iron complex prevents iron donation to iron-free chelators in samples[23]. The iron complexes of some chelators can also potentially interfere with the NTBI determination: with the NTA-method, the deferiprone-iron complexes are detected as 'NTBI' up to 1 week following drug cessation[17]. In principle, the bead assay can also detect such complexes of deferiprone[23], and indeed our findings here suggest that these are detected to a greater extent than with the NTA assay (**Figs 3G,H,I**).

Due to the high stability of ferrioxamine (1:1 iron(III):desferrioxamine) the bead-assay removes only negligible amount of iron from DFO-iron complexes[23]. Given this observation, it is difficult to explain the presence of two outliers in Figure 3G and perhaps the iron-

binding DFO metabolites should be considered. In the deferasirox-treated patients, NTBI is lower than in the other chelators using both assays but especially with the bead-assay, with values <1μM (**Fig 3F**). This may reflect superior NTBI removal with deferasirox or that patients in this group had 72h washout from chelators, allowing full clearance of deferasirox-iron complexes. These complexes may clear more rapidly than those of deferiprone which are measureable >1 week after the last chelation dose[17]. Thus in patients who are on regular chelation therapy, interpretation of NTBI values needs to be made with reference to the timing of sampling, particularly with the bead-assay which has high affinity for iron-deferiprone and iron-deferasirox complexes[23].

Clinical factors potentially affecting the detection levels of both NTBI assays were examined. Underlying diagnoses in themselves did not show significant differences between assays (**Table II**) but the extent of erythropoiesis did (**Fig S7A**): higher sTfR levels (and hence greater erythropoiesis and iron removal from transferrin) reduce NTA-NTBI, but not bead-NTBI values. As lower TfSat associates with decreased NTBI values due to NTA shuttling iron onto transferrin, clearance of transferrin-bound iron by enhanced erythropoiesis may increase iron shuttling by NTA and hence lower measured values. Negative prediction of the bias by TfSat suggests that the methods are differentially affected by TfSat, consistent with the methodological dependence of the NTA method on TfSat where NTA strips iron from transferrin, with NTBI overestimation rendering the bias lower. Likewise DFX negatively predicts the bias because it affects only the bead-NTBI (**Fig 3F**), and this is a key issue we are currently investigating. Normal status increases the bias by rendering NTA values negative (ApoTf). Chelation increases the difference because it increases the bead-NTBI more than the NTA-NTBI when detecting deferiprone-iron complexes. Splenectomy predicts NTBI by either method using multivariate analysis and also associates with higher NTBI (by 0.5 or 1.3μM, **Table II**). That splenectomy predicts bias is interesting while at the same time being a positive predictor of both methods separately, because it implies that bead assay detects more NTBI in splenectomised patients rather than that the NTA method detects less **(Fig S7B)**, suggesting it differentially affects the manner in which both methods may detect NTBI (splenectomy-dependent NTBI speciation differences). Higher NTA-NTBI in splenectomised TI patients was reported[36], as was a greater risk of myocardial iron deposition post-splenectomy[37]. The mechanism for higher NTBI post-splenectomy is unclear but we suggest may relate to erythrocyte destruction being diverted from the spleen to the bone marrow. With relatively hypoxic bone marrow environment, oxidation from Fe^{2+} to Fe^{3+} will have slower kinetics, hence slower iron binding to transferrin, and greater propensity to plasma NTBI formation. Similarly, higher levels of iron overload (SF or LIC) are associated with higher bead-NTBI than NTA-NTBI values, suggesting the bead method may detect

some iron species associated with iron overload that are relatively unavailable for capture using the NTA method.

Unlike the NTBI values by either method, the TfSat is notably predicted by diagnoses and ferritin in our cohort. In the explanation of the ferritin effect on TfSat but not on NTBI (the latter also reported recently[18]), we distinguished plasma iron compartment into the transferrin part, changing dynamically below 100% (TBI), and into NTBI part, *typically* present when transferrin is 100% saturated (TfSat not changing dynamically anymore). This means that ferritin as a predictor of TfSat marks the independent effect of the degree of tissue iron overload on the changes in the transferrin part of plasma iron compartment (i.e. TfSat increases when ferritin increases), but such independent effect on the level of NTBI, once TfSat is saturated, is absent. In other words if NTBI is considered in a continuum with TBI above the saturation point of Tf, it is evident that that plasma iron above the saturation point of Tf (NTBI) does not associate with ferritin (and therefore with iron overload) while the plasma iron on transferrin does. This is very interesting to us because it may suggest that regulation of serum iron varies by compartment: NTBI is less dependent for its generation and persistence on iron overload per se, and likely more dependent on other factors such as those that determine its removal rather than generation (tissue uptake – erythroid, hepatic)[5,38]. Finally, that sTfR does not predict TfSat but does negatively predict NTBI implies that the rate of transferrin off-loading in the marrow may affect the NTBI compartment without apotransferrin being detected peripherally[38].

In conclusion, we have identified that the TfSat in the blood sample affects values obtained with the NTA assay to a greater extent than with the bead assay. This results from iron donation to apotransferrin by NTA and/or stripping of iron from saturated transferrin by NTA. Neither of these effects is significant with the bead assay, which may therefore be more specific for true NTBI determination. These findings are consistent with first principles, namely differential access to transferrin iron of both methods, but other mechanisms may be involved, which require further systematic study. The presence of iron-chelate complexes in patients on chelation, particularly with deferiprone, increases values obtained with the bead assay more than with the NTA assay. Recent chelation history (minimum a week before blood sampling) needs accounting for when interpreting NTBI values obtained with either method. Other differences between the assays regarding the effects of splenectomy, levels of iron overload and endogenous erythropoiesis are consistent with both assays differentially accessing NTBI pools that vary with these clinical variables. Future work will need to identify whether NTBI values obtained with the bead assay are more clinically predictive of trends, such as myocardial iron deposition, than with the NTA assay. The effects of sample timing in relation to currently available chelation therapies need to be further defined with

both assays. We recommend using both methods in NTBI research since the NTA-NTBI and the bead-NTBI do not detect exactly the same species of NTBI, and that further research is necessary to describe NTBI speciation in greater detail before a recommendation can be made (if at all) which method should the researchers rely on.

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Statement of author contributions

Authors contributed as follows: conception and design of the study (MG, YM, JP, RH, SS, SF), data acquisition and collection (YM, MG, SF, SS), data analysis (MG, YM), data interpretation (MG, JP, RH), literature search (MG, YM, RH, JP), generation of figures (MG), writing of the manuscript (MG, JP, RH). All authors approved and reviewed the manuscript before publication and have read the journal's authorship statement.

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List of online supporting information

Supplementary material

1. Subgroup univariate analysis
2. Table S3
3. Figures S4, S5, S6, S7

Figures legends

Figure 1. Comparison of NTA and beads method for NTBI measurement. **(A)** NTA and beads (CP851) methods plotted for all samples using original scale. Correlation coefficient r=0.57 (0.44-0.68) p< 0.0001 **(B)** Agreement shown using Bland-Altman analysis of difference (ΔNTBI=NTA-CP851) vs mean of the two methods on data from panel A with bias -1.21±2.25µM and 95% LoA (limits of agreement) from -5.6 to 3.2µM. **(C)** Both methods for NTBI measurement plotted as in panel A but with negative values by both methods shown as zero (absent NTBI) Correlation coefficient r=0.6 (0.48-0.7) p<0.0001. **(D)** Agreement shown using difference vs mean Bland-Altman analysis of data in panel C, bias -0.56±2.1µM, 95% LoA -4.68 to 3.56µM.

Figure 2. Distribution of CP851-NTBI, NTA-NTBI, and transferrin saturation values with analysis of false-positive and false-negative NTA-NTBI values. **(A)** Comparing frequency distribution histograms of CP851-NTBI (red) and NTA-NTBI (black) in 135 pairs; frequency (number of values) of NTBI per interval of 0.5µM(bin size) is shown. **Inset** shows relationship between NTBI level and urea-gel TfSat for both methods: CP851-NTBI (red) and NTA-NTBI (black); best-fit linear regression slope differences (p<0.0001) with 100% TfSat points excluded from regression. **(B)** Plots of Tf saturation on x-axis (in 10% increments, bin center) against frequency of TfSat observations (y-axis) for NTBI values < or > 0µM. Left panel shows bead method and right panel NTA method. The TfSat distributions for NTBI values > 0µM are significantly different from those under 0µM by the NTA method only (Mann-Whitney test); at low TfSat negative NTBI values are significantly more likely by NTA method and at high TfSat positive NTBI values are more likely by NTA method. These differences are not apparent by the beads method. **(C)** Plot of NTA-NTBI values vs true-positive bead-NTBI values (above ULN=0.68µM - dashed line in Figure1A), size of the point reflects TfSat. Data in red-shaded box (below ULN) is shown in panel D. **(D)** Plot of NTA-NTBI vs true-negative bead-NTBI (red-shaded box in panel C corresponds to the data to the left of the dashed line in Figure 1A) with point size reflecting TfSat. Mean TfSat for NTA values >0µM was 93%, 19/36 were 100%, range 62-100%, median, 25th and 75th percentile: 100, 91, 100%. Mean TfSat for NTA values <0µM was 41.5%, 1/46 was 100%, range 11-100%, median, 25th and 75th percentile: 39.5, 31.5, 46%.

Figure 3

Figure 3. Effect of chelation on method agreement. **(A)**. Comparison of NTBI distributions in patients not chelated (upper panel) and chelated (lower panel), red symbols show the bead

CP851 assay and black symbols the NTA assay. **(B)** Comparison of NTA method vs bead method in patients with (black) and without (red) chelation, of the outliers marked only the chelation ones were excluded from analysis in CDE. **(C)** Comparison of TfSat distributions in chelated and unchelated patients seen in B, Mann-Whitney test. **(D)** Comparison of CP851-NTBI in cheated and unchelated patients seen in B, Mann-Whitney test **(E)** Comparison of NTA-NTBI in chelated and unchelated patients seen in B, Mann-Whitney test. **(F)** NTA method vs bead method in patients on deferasirox, no correlation (ns) **(G)** NTA method vs bead method in patients on desferrioxamine, correlation coefficient r=0.47 (0.01-0.77), p=0.05. **(H)** NTA method vs bead method in patients on deferiprone, correlation coefficient r=0.63 (0.34-0.81), p=0.0003. **(I)** NTA method vs bead method in patients on combination therapy of deferiprone and Desferrioxamine, correlation coefficient r=0.78 (0.3-0.95), p=0.0076.

Tables

Table I.

Table I. Patient diagnoses, patient number correlation within diagnoses and NTBI differences for groups with n>5.

Diagnosis	n	Correlations[1]	Grouped[2]	NTBI comparison[3]
Eβ-Thalassaemia (Eβ-thal)	44	0.68 (0.48-0.81)***	n=84, r=0.63	1.3 vs 1.0***
β-Thalassaemia Major (β-TM)	24	0.72 (0.44-0.87)***	(0.49-0.74)***	0.84 vs 0.82**
Thalassaemia Intermedia (TI)	11	0.5 (-0.14-0.85) ns		-0.06 vs 1.54 ns
Bart's Hb	3	N/A		N/A
α-Thalassaemia	2			
Hereditary HFE Haemochromatosis (HH)	9	0.64 (-0.04-0.92) ns	n=38, r=0.66	-0.04 vs -1.67 ns
Sickle Cell Disease (SCD)	6	0.54 (-0.36-0.92) ns	(0.47-0.79)***	-0.07 vs -2.11 ns
Congenital Sideroblastic Anaemia (CSA)	5			
Myelodysplastic Syndrome (MDS)	3			
Diamond-Blackfan Anaemia (DBA)	2			
Pyruvate Kinase Deficiency Anaemia	2			
Aceruloplasminaemia	2			
Ferroportin Disease	2			
Red Cell Aplasia	1	N/A		N/A
Sickle-E-β-Thalassaemia (SCD)	1			
Congenital Dyserythropoietic Anaemia	1			
β-Thalassaemia-Hb Malay anaemia	1			
Haemolytic Anaemia	1			
Spherocytosis	1			
Atransferrinaemia	1			
Normal Volunteers	13	0 (horizontal line)		-0.1 vs -2.75***

[1] Spearman correlation coefficient between NTA-NTBI and bead-NTBI, (95% CI), p value.

[2] Spearman correlation coefficient between NTA-NTBI and bead-NTBI, (95% CI), p value in all thalassaemia and all non-thalassaemia diagnoses.

[3] Comparison of median bead-NTBI vs NTA-NTBI, [uM], Wilcoxon test

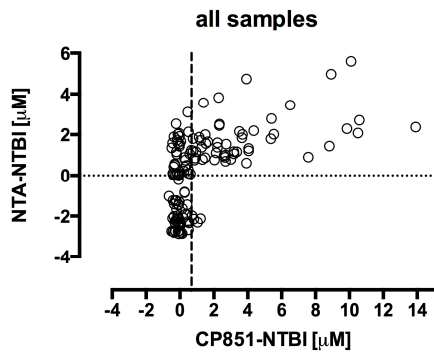p value <0.0001***, <0.001**, <0.01*, ns, not significant.

Table II

Table II. Multiple regression models for NTA-NTBI, CP851-NTBI, bias (CP851-NTA), and Transferrin Saturation.

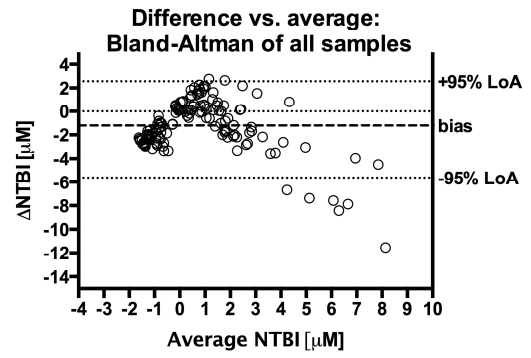| model	NTA-NTBI	CP851-NTBI	bias	TfSat
Adjusted r-square	0.75^{***}	0.26^{***}	0.24^{***}	0.42^{***}
n	101	120	117	100
Constant	$-3.89_{0.27}{}^{***}$	$-1.37_{0.59}{}^{*}$	$2.16_{0.62}{}^{**}$	$42.17_{5.01}{}^{***}$
TfSat	$0.056_{0.003}$ $(0.86)^{***}$	$0.028_{0.007}$ $(0.3)^{***}$	$-0.026_{0.007}$ $(-0.34)^{***}$	
Splenectomy yes=1	$0.51_{0.2}$ $(0.13)^{*}$	$1.32_{0.47}$ $(0.23)^{**}$	$0.86_{0.41}$ $(0.18)^{*}$	
Thalassaemia yes=1				$31.19_{5.67}$ $(0.52)^{***}$
Eβ-thal yes=1				$11.6_{4.75}$ $(0.21)^{*}$
MDS yes=1				$42.1_{15.3}$ $(0.21)^{**}$
CSA yes=1				$42.5_{10.4}$ $(0.33)^{***}$
DBA yes=1				$47.8_{15.2}$ $(0.24)^{**}$
SF [ug/L]				$0.006_{0.001}$ $(0.31)^{***}$
sTfR [nM]	$-0.003_{0.001}$ $(-0.12)^{*}$			
Chelation yes=1			$1.25_{0.45}$ $(0.27)^{**}$	
DFO+DFP yes=1		$2.29_{0.8}$ $(0.23)^{**}$	$1.59_{0.71}$ $(0.19)^{*}$	
DFO yes=1				
DFX yes=1			$-1.81_{0.61}(-0.26)^{**}$	
Normal yes=1			$1.42_{0.7}$ $(0.19)^{*}$	

Empty cell indicates that predictor was not significant in a particular model. Statistics for predictors (bold italics) are given as absolute regression coefficient, its standard deviation in subscript, adjusted regression coefficient in brackets, followed by significance of the predictor (***<0.0001, **<0.001, *<0.05). All other predictors insignificant (LIC, cardiac T2*, SGOT, SGPT, Ret, NRBC, Plt, WBC, Hb, Hct, MCV, MCH, MCHC, bilirubin, transfusion). Multiple linear regression on SPSS Version 22 was used.
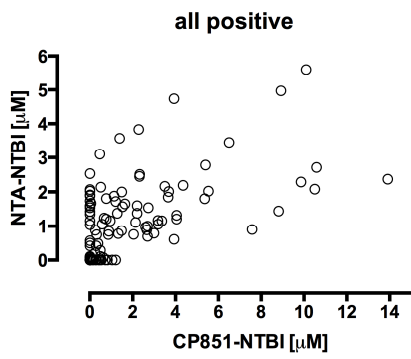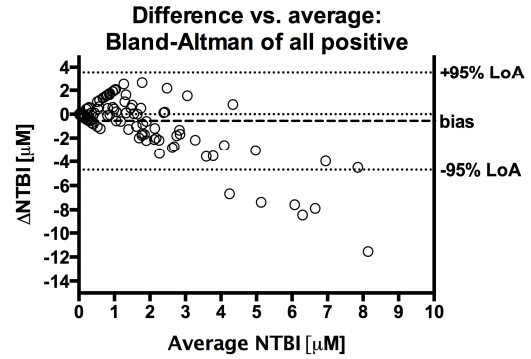
A comparing histograms

B Histogram of TfSat distribution around
CP851-NTBI=0μM   NTA-NTBI=0μM

p=0.84   p<0.0001

C positive NTBI by CP851 >ULN=0.68 μM

TfSat = 37±16.75%
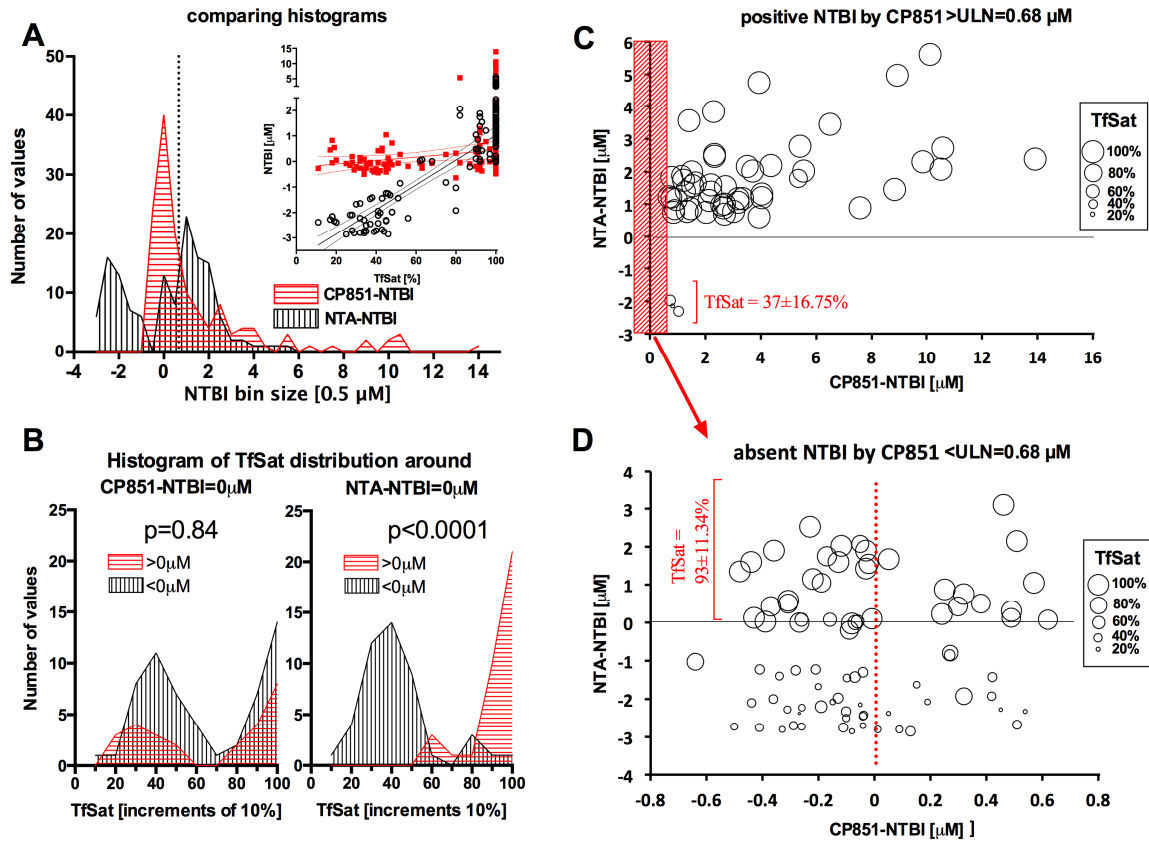
D absent NTBI by CP851 <ULN=0.68 μM

TfSat = 93±11.34%
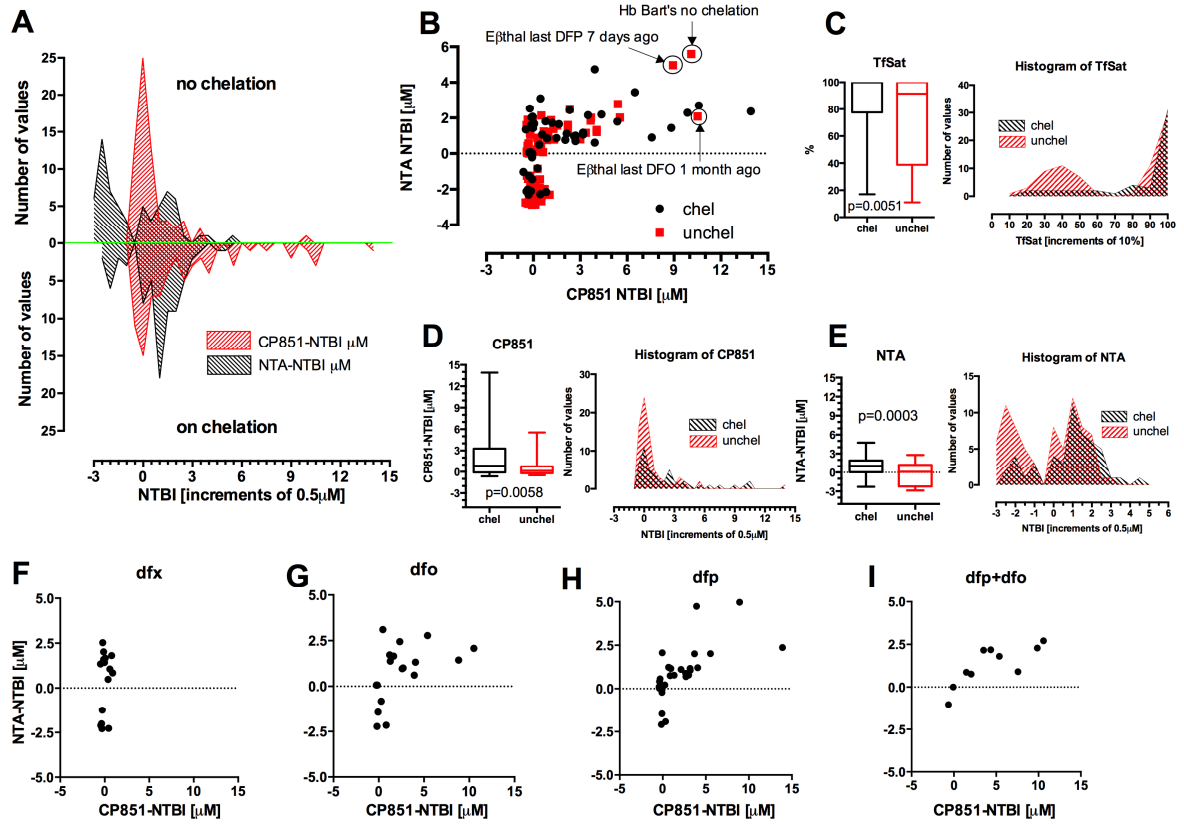
Brief Commentary

Background
NTBI is increasingly understood as a multi-speciated plasma iron pool, regulated separately from transferrin-bound iron and implicated in the complications of iron overload. The established NTA-NTBI method is not optimal for distinguishing transferrin bound iron from NTBI.

Translational significance
Here we compared a novel fluorescent bead method with the NTA method, across clinical diagnoses. The NTA assay underestimates or overestimates NTBI at low or high transferrin saturations respectively, which the bead assay being robust to effects of transferrin does not. The greater specificity of the bead assay should clarify links between raised NTBI levels and their clinical consequences.

Supplementary data

Subgroup univariate analysis

NTBI by underlying diagnoses

The relationship between both assays was broadly similar across different patient diagnoses although median bead-NTBI values were typically higher than NTA-NTBI for Eβ-thal, β-TM, SCD, HH and healthy controls (**Fig S4, Table I**). Significant correlations but poor agreement between both NTBI methods were seen for Eβ-thal, β-TM and all thalassaemias, with negative values by both methods treated as zero (**Fig S5, Table S3**). Other diagnoses were not sufficiently represented to draw correlation or agreement conclusions, however in non-thalassaemic group as a whole the correlation was significant (**Table I**). Normal control samples neither correlated nor agreed between the two assays but their bias could be corrected for (**Fig S5L**).

Transfusion

In transfused patients both methods correlated but non-transfused patients correlation was weak. Slopes were significant in both groups but only intercepts differed (**Table S3**). Significant effect of transfusion seems to be limited to high TfSat as only samples with TfSat>90% show significant positive slope among transfused patients (**Fig S6**). Bead-NTBI was higher than NTA-NTBI in transfused and untransfused patients (**Table S3**).

Erythropoiesis

High and low erythropoietic states were judged by soluble transferrin receptor (sTfR) levels above (high) and below (low-normal) 28.1nM (ULN). NTBI methods correlated, the slopes were significantly positive but differed from one another, for high and low-normal erythropoietic states. The NTA assay detected less NTBI relative to the bead assay for patients with high erythropoiesis compared to those with low-normal levels of erythropoiesis (**Table S3, Fig S7A**). Since a high erythropoietic rate will increase iron clearance from transferrin, this is predicted to free up iron binding sites on transferrin thus increasing iron shuttling onto these sites and hence lower levels with the NTA assay.

Splenectomy

In patients with and without splenectomy, the methods correlated well with positive slopes that differed significantly: bead-NTBI being higher, and the difference between methods being greater in splenectomised than un-splenectomised patient (**Fig S7B, Table S3**).

Storage iron

For high- and low-ferritin patient groups (above or below 1500 µg/L), methods correlated well with significantly different positive slopes. There was greater discrepancy between NTBI levels in the high ferritin than the low ferritin group (**Fig S7C, Table S3**): for similar CP851-NTBI values, NTA method gives lower NTBI values in the former and higher in the latter group above about 2 µM bead-NTBI threshold. Methods were also compared in LIC groups below and above 12mg/gdw (SF=1500ug/L corresponds in our study to LIC=12mg/gdw) showing medium correlation and significantly different positive slopes. In high LIC group, discrepancy between methods was greater (**Fig S7D, Table S3**). NTBI differences were insignificant on direct comparison: not all patients had LIC available (n=35 vs n=109 for ferritin), so comparison may be underpowered to detect differences. Taken together these findings show that at high storage iron (LIC or SF) the extent to which bead-NTBI values exceed NTA-NTBI is increased.

Table S3

Table S3. Subgroup analysis: correlations, slope comparison and NTBI differences between transfusion, erythropoiesis,

splenectomy, storage iron and chelator subgroups (p-value <0.0001***, <0.001**, <0.01*, ns, not significant).

Subgroup[1]	Correlation[2]	Slope[3]	test[4]	NTBI comp.[5]
all samples	0.61 (0.49-0.71), ***			1.5±2.59μM vs 0.94±1.13μM, *
transfused	0.57 (0.4-0.7), ***	0.31 (0.21-0.41), ***	ns, int**	2.04±2.99μM vs 0.76±1.56μM, ***
untrasfused	0.31 (0.02-0.55), *	0.83 (0.05-1.6), *		0.14±0.71μM vs -0.83±1.91μM, **
high sTfR	0.63 (0.48-0.74), ***	0.25 (0.18-0.31), ***	*	1.8±2.9μM vs 1.1±1.16μM, *
low-normal sTfR	0.6 (0.28-0.8), **	0.83 (0.36-1.29), **		0.44±0.83μM vs 0.7±1.13μM, ns
splenectomy	0.5 (0.24-0.69), **	0.14 (0.06-0.21), **	***	2.97±3.7μM vs 1.26±1.02μM, **
no splenectomy	0.68 (0.55-0.79), ***	0.44 (0.33-0.54), ***		0.84±1.7μM vs 0.73±1.1μM, ns
SF >1500ug/L	0.55 (0.32-0.72), ***	0.17 (0.1-0.25), ***	*	0.82±1.66μM vs 0.71±1.03μM, ns
SF <1500ug/L	0.65 (0.47-0.78), ***	0.41 (0.280.54), ***		2.83±3.45μM vs 1.49±1.07μM, *
LIC>12	0.77 (0.48-0.91), ***	1.05 (0.6-1.49), ***	*	0.45±0.96μM vs 0.86±1.31μM, ns
LIC<12	0.66 (0.24-0.87), *	0.42 (0.14-0.69), *		0.63±1.67μM vs 1.19±1.06μM, ns

[1] conditions where bead-NTBI and NTA-NTBI were compared, [2] Pearson correlation coefficient (95% CI), p value, [3] Slope (95% CI), p value,

[4] comparison of slopes p-value or when ns, the intercept (int) comparison p-value, [5] mean NTBI comparison CP851 vs NTA (95% CI), t-test p
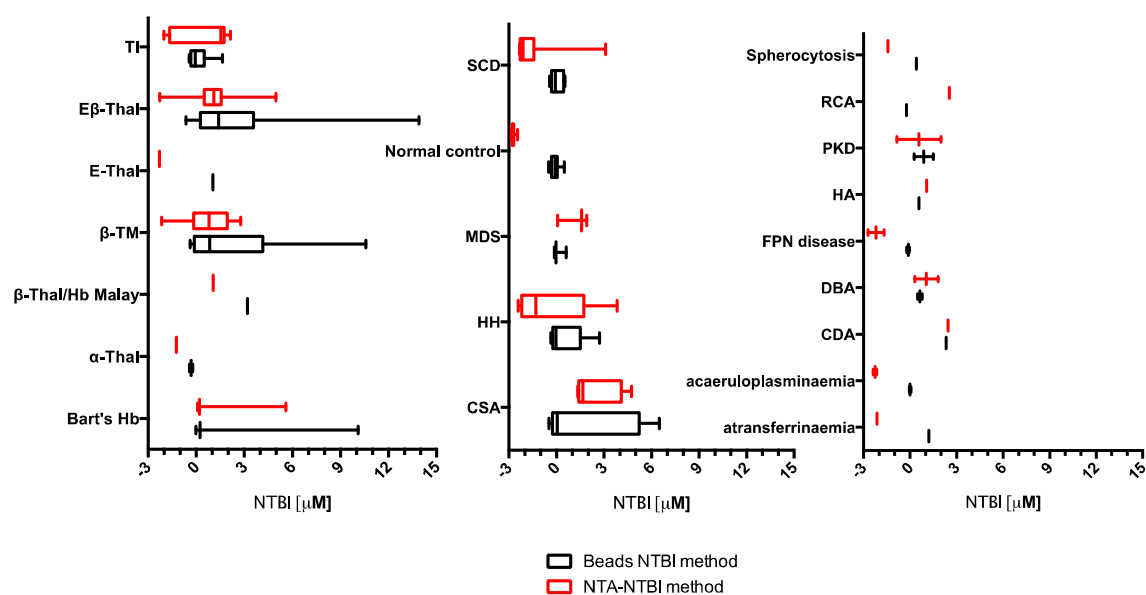
Figure S4

Figure S4. Box and whisker plot (range) of NTBI in healthy controls and various conditions of iron overload using a standard analytical method (NTA-NTBI) and the proposed bead-based NTBI method.
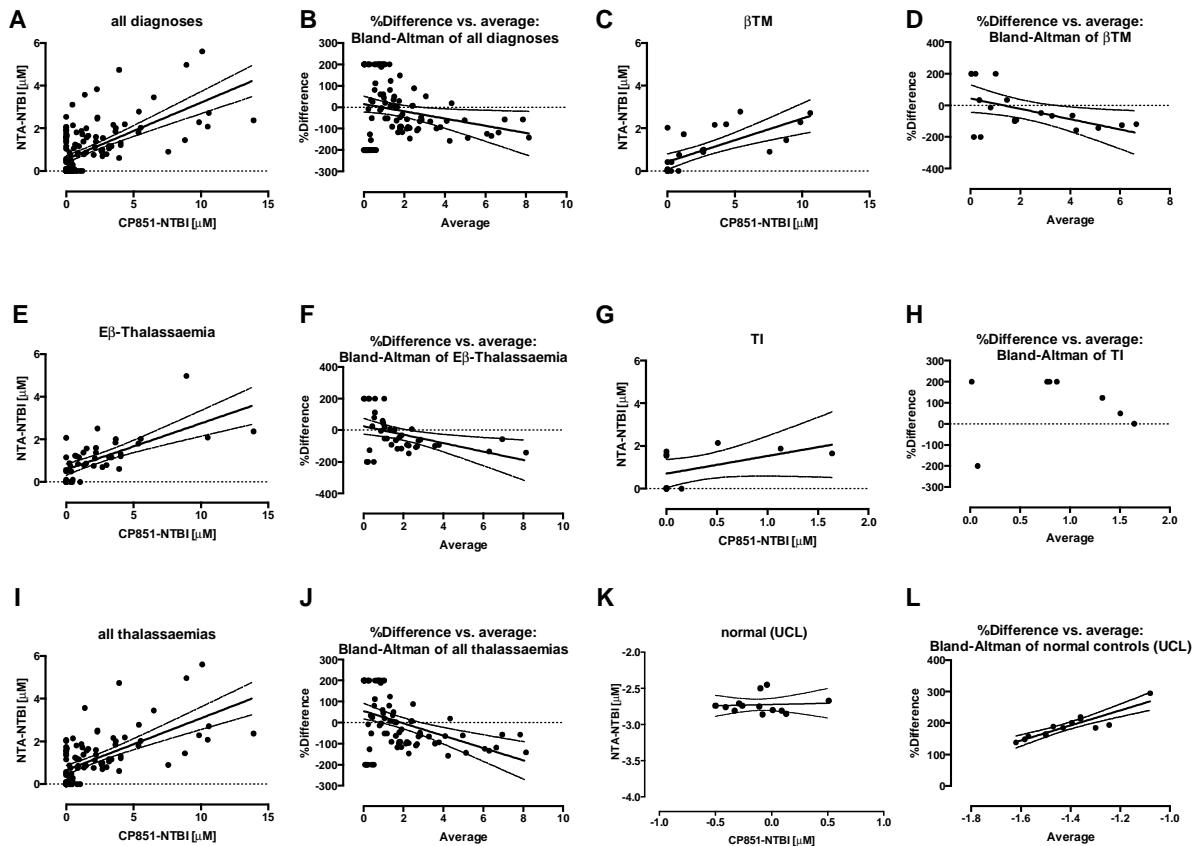
Figure S5

Figure S5. Comparison and Bland-Altman analysis of agreement of NTA and CP851-NTBI methods in different diagnosis groups. Negative values by both methods have been treated as zero. **(A-B)** all diagnoses; slope p<0.0001. **(C-D)** beta-Thalassaemia Major; slope p<0.0001. **(E-F)** E-beta-Thalassamia; slope p<0.0001. **(G-H)** thalassaemia intermedia; slope not significant. **(I-J)** all thalassaemias; slope p<0.0001. **(K-L)** normal volunteers; slope not significant. All Bland-Altman analyses show lack of acceptable agreement except for the normal samples where the low scatter systematic bias could be corrected for using the formula (239.1±35.12[%]*avgNTBI[uM]+527.3±50[%])(27).
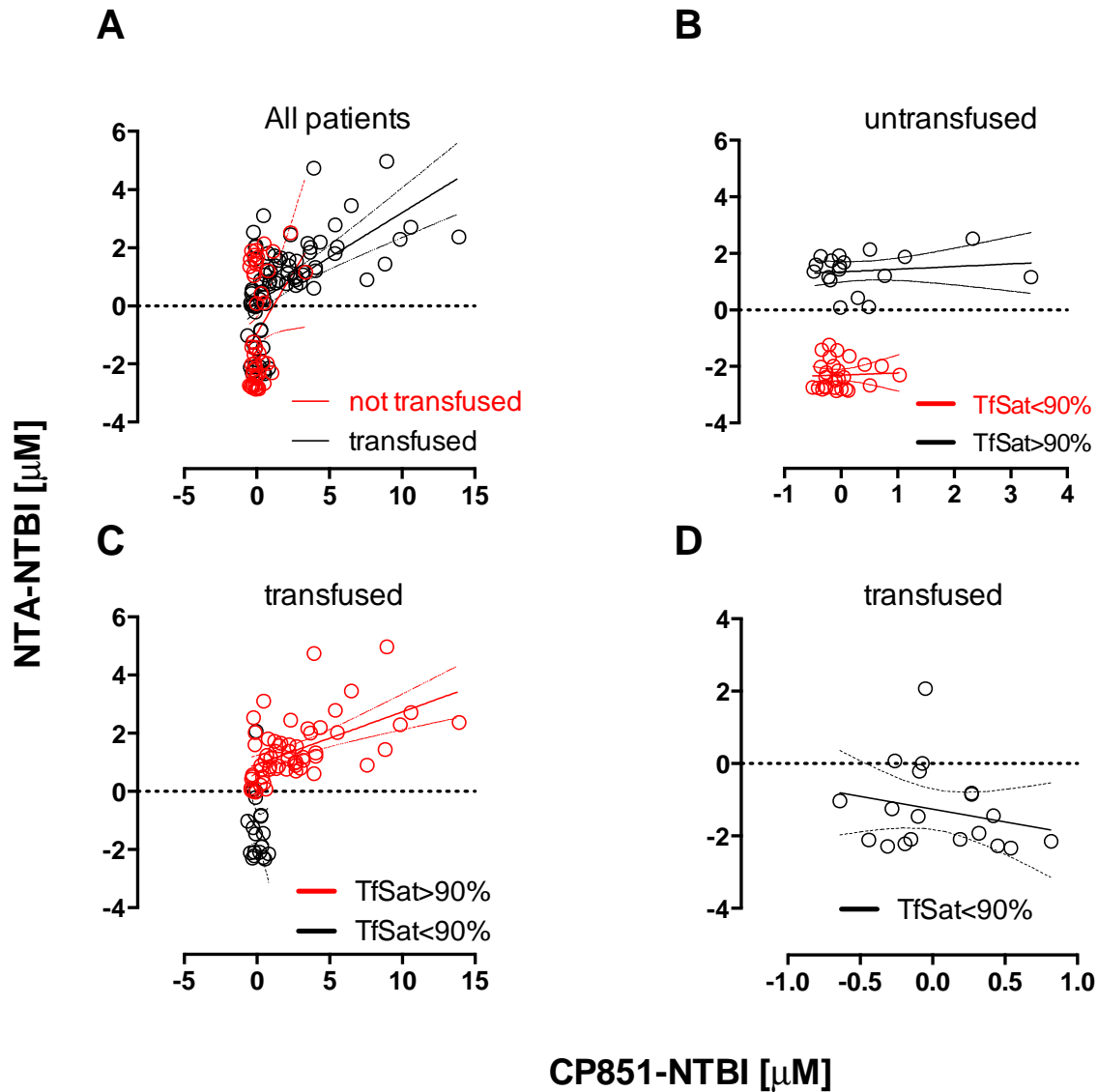
Figure S6

Figure S6. The effect of transferrin saturation on method agreement in transfused and untransfused patients. (**A**) Plot of both NTBI methods in transfused (black) and untransfused patients (red). (**B**) Plot of both NTBI methods in untransfused patients (red in panel A). Samples with TfSat>90% are now shown in red and those with TfSat<90% in black. (**C**) Both NTBI methods plotted in transfused patients (black in panel A), here further grouped according to TfSat in sample: with significant slope in TfSat>90% (red, p<0.0001) and insignificant in TfSat<90% (black). (**D**) Both methods plotted for transfused patients with TfSat <90% show insignificant slope (black symbols from panel E magnified).
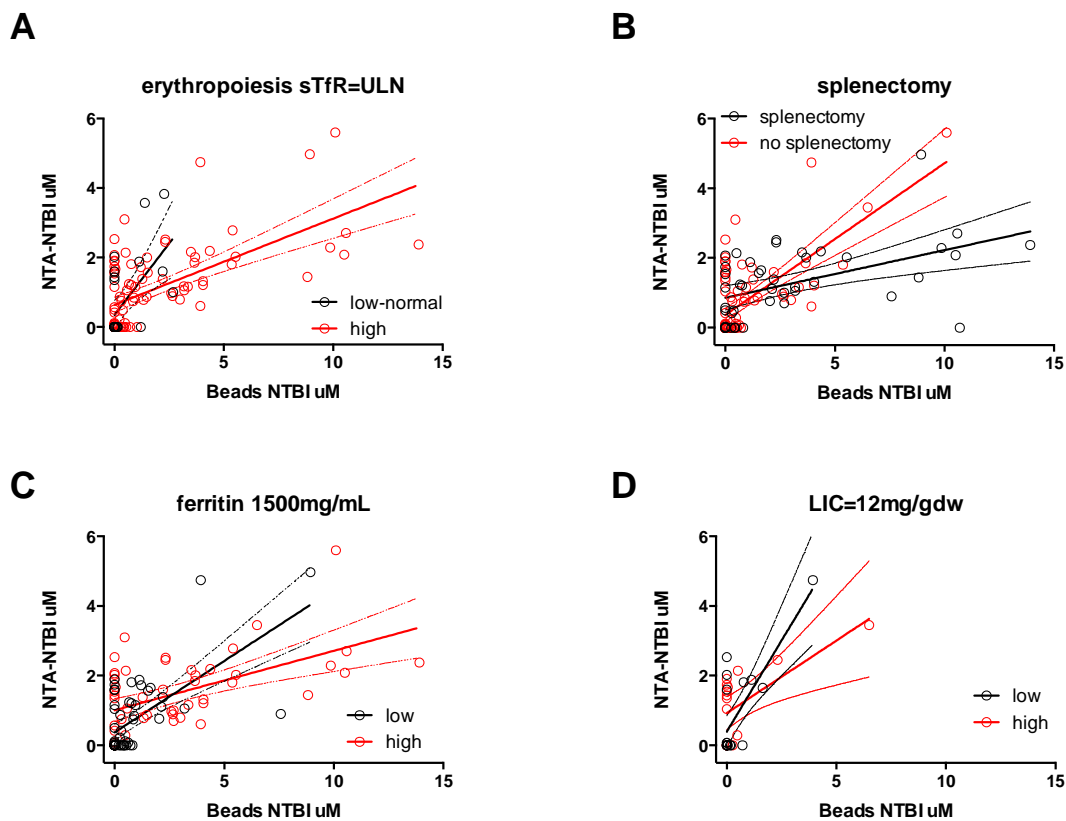
Figure S7

Figure S7. Comparison of NTA-NTBI and CP851-NTBI methods in groups with different erythropoietic, spleen, ferritin and LIC status. **(A)** Slope comparison between high and low-normal erythropoietic status defined by soluble transferrin receptor: both slopes are significant (0.25±0.03, p<0.0001 and 0.82±0.22, p=0.001) and different (p=0.01); patients mean sTfR (n=117) was 93.46±68.1nM (median 88.24, IQR 30.1-133.2, range 5.38-332.5) **(B)** Slope comparison between patients with and without splenectomy: both slopes are significant (0.14±0.04, p=0.0005, and 0.44±0.05, p<0.0001) and different (p<0.0001). **(C)** Slope comparison between low and high ferritin groups (1500ug/L): both slopes are significant (0.41±0.06 and 0.17±0.04, both p<0.0001) and different (p<0.0013). **(D)** Slope comparison in low and high LIC groups (12mg/gdw): both slopes significant (1.05±0.21, p=0.0001 and 0.42±0.13, p=0.006) and different (p=0.015).