Second International Round Robin for the Quantification of Serum Non-Transferrin-Bound Iron and Labile Plasma Iron in Patients with Iron-Overload Disorders


Haematologica 2015 [Epub ahead of print]

doi:10.3324/haematol.2015.133983

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Second International Round Robin for the Quantification of Serum Non-Transferrin-Bound Iron and Labile Plasma Iron In Patients with Iron-Overload Disorders

Running head: Round Robin for NTBI and LPI

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*This work is dedicated to the memory and in honor of Renzo Galanello, who was instrumental for the study until the end of his career.
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**Key words**: NTBI; Non-transferrin-bound iron; LPI; labile plasma iron; Round robin; Iron overload.

**List of abbreviations in order cited**: NTBI, non-transferrin-bound iron; LPI, labile plasma iron; HH, hereditary hemochromatosis; Thal, β-thalassemia; MDS, myelodysplastic syndrome; SCD, sickle cell disease; TSAT, transferrin saturation; RR, round robin; TD, transfusion dependent; CH, receiving iron chelation; Hb, hemoglobin; MCV, mean corpuscular volume; TIBC, total iron binding capacity; NTA, nitrilotriacetate; DCI, directly chelatable iron; LC-ICP-MS, liquid-chromatography inductively-coupled-plasma mass spectrometry; ROS, reactive oxygen species; TBA, thiobarbituric acid; DHR, dihydorhodamine; eLPI, enhanced LPI; LLOD, lower limit of detection; ULOD, upper limit of detection; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; CRP, C-reactive protein; LDH, lactate dehydrogenase.
Abstract

Non-Transferrin Bound Iron and its Labile (redox active) Plasma Iron component are thought to be potentially toxic forms of iron originally identified in the serum of patients with iron overload. We compared 10 worldwide leading assays (6 Non-Transferrin Bound Iron and 4 Labile Plasma Iron) as part of an international inter-laboratory study. Serum samples from 60 patients with 4 different iron-overload disorders in various treatment phases were coded and sent in duplicate for analysis to 5 different laboratories worldwide. Some laboratories provided multiple assays. Overall, highest assay levels were observed for patients with untreated hereditary hemochromatosis and β-thalassemia intermedia, patients with transfusion-dependent myelodysplastic syndromes and patients with transfusion-dependent and chelated β-thalassemia major. Absolute levels differed considerably between assays and were lower for Labile Plasma Iron than for Non-Transferrin Bound Iron. Four assays also reported negative values. Assays were reproducible with high between-sample and low within-sample variation. Assays correlate and correlations were highest within the group of Non-Transferrin Bound Iron assays and within that of Labile Plasma Iron assays. Increased transferrin saturation, but not ferritin, was a good indicator for the presence of circulating Non-Transferrin Bound Iron forms. The possibility of using Non-Transferrin Bound Iron and Labile Plasma Iron measures as clinical indicators of overt iron overload and/or of treatment efficacy would largely depend on the rigorous validation and standardization of assays.
Introduction

The predominant forms of iron present in living entities are associated with proteins such as transferrin in the major circulating fluid and haem and ferritin in cells. However, the iron binding capacity of the iron transport protein transferrin can be surpassed when a substantial amount of iron enters the circulation due to excessive iron absorption from the diet or an outpour of iron from cell stores. In these conditions non-transferrin-bound-iron (NTBI; circulating iron not bound to transferrin, ferritin or haem) appears in the plasma.\textsuperscript{1-5} Plasma NTBI is apparently comprised of several subspecies, which may be classified either by their chemical composition, chemical reactivity and susceptibility to chelation.\textsuperscript{6-15} As for the chemical composition NTBI is heterogeneous and thought to consist of several circulating isoforms, that is Fe(III) bound to albumin and citrate and potentially to acetate, malate and phosphate.\textsuperscript{6-8,14} Of these, citrate has the highest affinity for Fe(III), and under physiological conditions two isoforms dominate, i.e. monomeric and oligomeric Fe(III) complexes.\textsuperscript{8,16} The fraction of plasma NTBI that is redox-active and chelatable is designated labile plasma iron (LPI).\textsuperscript{12,13}

Iron complexes assumed to represent NTBI have been shown experimentally to be taken up by susceptible cell types, including hepatocytes, cardiomyocytes and pancreatic islet cells, with consequent oxidant injury.\textsuperscript{5}

Imbalances in iron homeostasis are responsible for a variety of disorders. Excess iron accumulates in the circulation and tissues of patients with hereditary hemochromatosis (HH), iron-loading anemias (β-thalassemia (Thal) major and intermedia), myelodysplastic syndromes (MDS) and sickle-cell disease (SCD) after transfusion.\textsuperscript{2,3,5} To prevent iron-induced tissue damage, detection of impending iron toxicity is needed before complications develop and become irreversible.
Currently, the most widely adopted method for the detection of iron overload is the measurement of serum ferritin, occasionally combined with transferrin saturation (TSAT). However, it is well known that as an acute phase reactant, serum ferritin levels are influenced by factors like inflammation and liver disease and therefore they are not universally representative of toxic parenchymal iron overload. Moreover, with the introduction of MRI T2* for the assessment of tissue iron overload, it became also clear that organs such as the heart and endocrine glands load iron differently compared to the liver and non-commensurately with serum ferritin. Studies of plasma NTBI in Thal major patients suggest that NTBI may be an important early indicator of extra-hepatic iron toxicity. Studies in patients with various iron-loading disorders have shown a reduction in NTBI and LPI upon phlebotomy and chelation therapy that is associated with an improved prognosis. Therefore, NTBI and LPI assays are promising as therapeutic targets and for the evaluation of iron overload and the efficacy and compliance of iron-lowering therapies.

Due to the complexity and potential clinical importance of NTBI, several assays have been developed for its detection. In our previous Round Robin 1 (RR1) we found that NTBI values of patients with HFE-related hemochromatosis differed considerably between the various assays. We concluded that NTBI assays were insufficiently standardized and the most pertinent assay for clinical applications remained uncertain. Since this RR1 for NTBI, novel assays have been published and made available for use. Therefore, and as a stepping stone in the path to defining the clinical utility of these assays, the aims of our study were to i) update RR1 and ii) increase our understanding of the various NTBI and LPI levels measured by the current leading analytical assays in four different groups of iron overloaded patients (HH, Thal, MDS, SCD) undergoing various treatments (phlebotomy, iron chelation, red blood cell transfusion). More specifically, in these patient populations, we aim to 1) establish correlations between assays, 2) establish levels of reproducibility of each of the
assays, 3) assess levels of the NTBI fraction consisting of iron citrate, and 4) correlate assays with other established parameters of iron overload (e.g., TSAT and serum ferritin).

Methods

Study design and participants

We compared 10 different assays (5 NTBI, 1 NTBI isoform-specific and 4 LPI) performed in 5 different laboratories worldwide. Serum samples (n=60) were collected from patients with 4 iron overload disorders in various treatment phases (TD, transfusion dependent; CH, receiving iron chelation) making a total of 10 different patient and treatment groups. Sample collection was performed with approval from local IRBs and or conforming to the code for proper secondary use of human tissue. More detailed information on collection of samples and laboratory analysis is described in the online supplemental information.

NTBI and LPI assays

The participating NTBI/LPI assays were divided into 3 different assay groups: i) 5 NTBI assays (N1-N5) ii) 1 NTBI isoform-specific assay (N6) and iii) 4 LPI assays (L1-L4) (supplemental table 1). The test principles of the NTBI assays can be divided into two subgroups. The first subgroup (N2-4) consists of the chelation-ultrafiltration-detection approach based on the prior mobilization of serum NTBI by weak iron-mobilizing chelators such as nitrilotriacetate (NTA) at 80 mM. The chelated NTBI is separated from transferrin-bound iron by ultrafiltration and detected by colorimetry or HPLC.26-28 The second subgroup of NTBI assays (N1 and N5) comprises the measurement of directly chelatable iron (DCI). In these assays NTBI is mobilized and detected in the same reaction mixture by iron-binding probes such as fluorescently-labeled desferrioxamine and the hexadentate pyridinone chelator (CP851) attached to a fluorescent probe.6,10,15 In the latter assay the CP851 was bound to beads and the fluorescent signal from the bead-bound chelator was differentiated from the
non-specific plasma signals by flow cytometry.\textsuperscript{6,15} None of the NTBI assays used a cobalt compound to block unsaturated transferrin. The NTBI isoform-specific method N6 measures the sum of the oligomeric and monomeric iron (III)-citrate complexes of NTBI. This methodology consists of gel filtration chromatography by HPLC to separate the individual components of NTBI by mass, followed by quantification of iron by inductively coupled-plasma mass spectrometry (LC-ICP-MS).\textsuperscript{6}

The LPI assays (L1-4) measure the redox active component of NTBI, a potentially toxic species. Assay L1 used bleomycin, which forms a complex with DNA and redox active ferrous iron. When ascorbate is added, reactive oxygen species (ROS) are generated which degrade the deoxyribose moieties of the DNA in a site-specific reaction. These degradation products bind to thiobarbituric acid (TBA) to give a pink chromogen for quantification.\textsuperscript{25} The assays L2, L3 and L4 used a reducing agent (ascorbate) and an oxidizing agent (atmospheric O\textsubscript{2}) to generate Reactive Oxygen Species (ROS) from endogenous oxidants and labile catalytic iron. ROS were detected by an oxidation-sensitive probe (dihydrorhodamine (DHR)). Comparison of the generated fluorescence in the presence and absence of an iron chelator (deferiprone or deferrioxamine) makes the assay specific for iron, which can then be measured from a standard curve generated from known iron concentrations.\textsuperscript{12,29} For the extraction of iron from NTBI, assay L4 used 0.1 mM NTA to capture the mobilizer-dependent form of LPI (enhanced or eLPI).\textsuperscript{11,29}

Detailed information on statistical analysis is described in the online supplemental information.
Results

Patient characteristics

Patient characteristics by disease and treatment groups are shown in table 1. Serum ferritin levels were most elevated in Thal major TD & CH, MDS TD and SCD TD and in HH patients at presentation (HH naive). The ferritin levels in HH patients decrease after phlebotomy. TSAT levels were similar for the two different TSAT methodologies used and were highest in Thal major TD & CH (median 100%). Median TSAT was also very high (>94%) in patients with naive HH, MDS TD and Thal intermedia naive, and less increased (>51%) in Thal major naive, in the depletion phase of phlebotomies in HH patients, in MDS, refractory anemia with ringed sideroblasts (RARS)/refractory cytopenia with multilineage dysplasia (RCMD)-RS and in SCD TD, and within reference range (<45%) for most patients with naive SCD and HH patients in the maintenance phase.

Assay levels and limits of detection

Absolute NTBI and LPI levels differed considerably between assays (table 2). Overall, the NTBI assays (N1-N4, N6) measured ~2-30 times higher concentrations compared to the LPI assays (L1-L4), in agreement with the concept that NTBI assays measure total circulating NTBI, whereas LPI assays specifically measure the redox active fraction. Nonetheless, major differences in absolute values were found within the group of NTBI and LPI assays. For example, both N1 and N5 measure DCI but levels are much higher for N1 than for N5. In fact, N5 levels were greater than the cut-off of zero in only 8% of the samples (n=5). This assay was therefore excluded for further analysis.

As expected, levels of assay L4 that measures the forms of LPI that are detectable in the presence of NTA (designated “eLPI”), are higher than those obtained by assay L3 that uses a methodology that is identical to L4 except that NTA is not added.
Assays differed widely in the use of predefined detection limits (supplemental table 1). Four out of 10 participating methods reported negative values: i.e. N1, N2, L1 and L2 in 20%, 60%, 53% and 25% of the samples, respectively. For assays N5, L3 and L4, 92%, 70%, and 28% of the samples, respectively, were measured as ≤ 0 but were reported as 0 (zero) NTBI or LPI. For 2 of these assays, L3 and L4, also an upper limit of detection (ULOD) of 2.2 µmol/L was employed since the assay is non-linear above this concentration. Three assays (N3, N4, N6) measured positive values only. Two of them (N3 and N4) applied a lower limit of detection (LLOD) of 0.60 µmol/L and 0.87 µmol/L with reported results < LLOD in 42% and 32% of the samples, respectively.

Reproducibility

Assays showed a high contribution of the between-sample variance to the total variance indicating that the assays are able to identify different NTBI/LPI concentrations in the various patient groups (table 3). The relatively low between-sample variance of assay N4 could be attributed to the relatively high residual variance (33%) due to some outliers. For all assays, the contribution of the within-sample variance (within day and between-day) to the total variance was relatively low (0-2.2%), indicating good reproducibility.

Comparison between assays

Overall, assays correlate well and correlations were highest within the same group of NTBI or LPI assays (table 4, supplemental figure 1). The NTBI and LPI assays with the highest mutual Spearman’s correlation were N2 and N3 (r_s=0.88), N3 and N4 (r_s=0.86), N2 and N4 (r_s=0.85), L3 and L4 (r_s=0.77), and L2 and L3 (r_s=0.74). Spearman correlations of N6 with the other assays were all <0.10.
Remarkably, the Spearman correlation of LPI assay L1, using bleomycin, with NTBI assays was higher compared to the LPI assays. In contrast, NTBI assay N1 showed a better Spearman correlation with LPI assays than with NTBI assays (table 4).

In Figure 1 the Bland–Altman plots are shown of a selection of 9 graphs with the highest mutual correlation or a specific correlation of interest. Two patterns can be observed in the plots: i) if the scales of one variable are small compared to the other one, the straight line pattern occurs (e.g. in the N2-L1 plot), ii) if cut-off values are used in one variable and not in the other variable, a straight line occurs in combination with a scattered pattern (e.g. the N2-N3 plot).

**Discrimination of disorder and treatment groups by the assays**

Table 2 shows the results obtained by the 9 assays in the 10 different disease and treatment groups. Using a linear mixed model, we found that all methods were suitable to discriminate, at least on a group level, between some different types of iron overload disease (p<0.05), except for the NTBI isoform-specific assay N6 (p=0.268) (supplementary table 2). Overall, assays were best in discriminating LPI and NTBI from patients with highest TSAT levels (Thal major TD & CH, Thal intermedia, MDS TD and untreated HH) from those of the diseases with lower TSAT (SCD naive, SCD TD and HH maintenance; table 2 and supplemental Table 2).

**Comparison of assays with TSAT and ferritin**

The assays show either a hyperbolic relationship or a threshold effect with TSAT, regardless of the underlying etiological condition, NTBI concentrations increase sharply when TSAT levels exceeded 70%, NTBI is sporadically observed in samples with TSAT lower than 70% and LPI is found essentially when TSAT exceeds 90% (Figure 2). More specifically, at TSAT
> 90%, the presence of LPI and NTBI may be taken for granted for N1, N2, N3, N4, N6, L1 and L4 since for these assays the proportion of samples where NTBI or LPI is found is 95.7 or 100%. At TSAT > 70%, these proportions are somewhat lower, but for N3 and N6 already 100% (Supplemental Table 3).

There is no relationship between serum ferritin and either LPI or NTBI measured in any of the assays (data not shown).

**Discussion**

We compared the performance of ten different NTBI and LPI assays in patients with 4 different iron-overload diseases in various treatment phases. Nine out of 10 assays reported an assay value above zero in ≥30% of the samples. These 9 methods exhibited a high between-sample variation and low within-sample variation indicating their suitability to identify different serum NTBI and LPI concentrations in patients with iron-overload. Overall, results correlated both within the group of five NTBI assays and in the group of the four LPI assays, but between group correlations were also present. Nonetheless, absolute assay levels differed considerably between assays, with lowest levels for LPI methods. Overall, assay levels correlated with TSAT but not with ferritin concentrations and iron-overload diseases with the highest TSAT also had the highest levels of NTBI and LPI.

Some of the differences in absolute values between the assays can be attributed to differences in the assay principles that result in the measurement of different NTBI entities, e.g. NTBI, DCI, NTBI-iron citrate, LPI and eLPI. In addition, minor variations in analytical procedures between methods aiming at the measurement of the same entities (N2-N4 or N1 and N5 or L2 and L3) may also have contributed to between-assay variation in absolute values. For instance, it has recently been shown that for NTA based assays that use filtration, centrifugation at extremely high speed can lead to overestimation of the NTBI concentration.
due to passage of transferrin-bound iron through the filter membrane. Conversely, when centrifuging at lower speed, iron-NTA complexes do not pass the membrane, leading to underestimation.\textsuperscript{31} The LPI assays also mutually differ in reagents and buffers. The presence of residual chelator or chelates could contribute to higher values in some assays, such as the bleomycin-based LPI (L1) assay and the NTA-filtration assays (N2-N4), but not in others.\textsuperscript{11} Furthermore, iron-contamination may also be a source of variation in absolute assay concentrations.\textsuperscript{32} It could contribute to the reported negative values by mimicking free circulating iron. Rather than registering as an increase in NTBI, this contaminant free iron can shuttle from the iron chelator NTA to vacant binding sites on transferrin.\textsuperscript{27, 31, 33} Thus samples containing unsaturated transferrin may have lower NTBI values compared to standards where no transferrin is present. In principle, this can result in underestimation of NTBI and negative values. This iron shuttling can be prevented by saturating transferrin with cobalt(III) prior to addition of the NTA.\textsuperscript{34} Still, others have reported that the addition of cobalt (III) may result in substantial iron-contamination that could account for the rise in NTBI even in normal individuals.\textsuperscript{32} Finally, for the iron chelator NTA applied in 3 NTBI assays, the use of 80 mM was shown by some authors to remove 1-8% of the iron bound to transferrin in case of elevated transferrin saturation, thus leading to overestimation of NTBI measurements.\textsuperscript{27, 31} By contrast, at physiological concentrations of serum iron in control subjects others report no mobilization of iron from transferrin under these conditions.\textsuperscript{28}

NTBI is thought to consist of Fe(III) bound to several ligands. Since iron bound to citrate is likely to be the most prevalent isoform of NTBI in most conditions, a LC-ICP-MS assay for the sum of these iron (III)-citrate complexes of NTBI was developed and included as N6 in the current Round Robin.\textsuperscript{6} The assay was reproducible and discriminated between samples, but assay levels did not correlate with other assays and could not discriminate between any of the disorders. Clearly, this method may need optimization, but if successful, we anticipate that
quantification of NTBI subspecies could provide the basis for further studies on their toxicity. Different methods may not be equivalent at measuring these subspecies. It has been postulated that the abundance of each subspecies varies with the type and degree of iron-overload disease.⁷,¹¹ Although this possibility is still under investigation, it implies that specific assays might be required for different chemical species of NTBI.⁶,⁷,¹¹,¹³ Overall the assays correlated well. Mutual correlations between the assays were found to be highest in methods based on a similar principle (i.e. in the group of NTBI assays and the group of LPI assays). We observed two unexpected correlations: first, despite the chelator-based principle of N1, correlation of N1 with LPI assays exceeded its correlation with other NTBI assays. A possible explanation could be the kinetics of access of CP851 to redox active NTBI.⁶,¹²,¹⁵,²⁷ Secondly, even though assay L1 is based on binding of the antibiotic bleomycin to redox-active iron and DNA, its results correlated slightly better with most NTBI assays than with LPI assays. Bleomycin-detectable NTBI might represent a larger redox active fraction of NTBI than LPI, in equilibrium with a larger, still uncharacterized pool of NTBI.

Differences in the range of reported values as well as in absolute values, have highlighted the urgent need for thorough method-validation protocols, standardization and consensus on how to best report before introduction for clinical use. Four out of 10 assays report negative values and as such provide information on the residual iron-binding capacity of transferrin. Other assays report 0 (zero) for results ≤0, or <LLOD for results that do not significantly differ from 0. It remains to be determined which reporting strategy is clinically more useful.

Importantly, both LPI and NTBI assays showed a relationship with TSAT, irrespective of the type of iron-overload disease. NTBI and LPI were essentially found above certain thresholds of TSAT, lower for NTBI (~70%) than for LPI (~90%). A comparable pattern was previously reported for LPI assays that were similar to assays L1-L3 for patients with various
conditions.\textsuperscript{2, 3, 35, 36} Altogether, our findings corroborate previous reports in HH and Thal patients that TSAT levels of around 70% can be considered as a threshold for the presence of potentially toxic iron species.\textsuperscript{2, 3} Nonetheless, because a single measurement only reflects the labile iron species present during the previous 24-48 hours, repetitive measurements are imperative to assess this potential risk of impending NTBI toxicity.\textsuperscript{18}

The majority of the assays of the current RR have given useful insights into the efficacy of iron chelation in transfusional siderotic patients and of phlebotomy in patients with hereditary hemochromatosis.\textsuperscript{2, 3, 9, 13, 19-22, 37-40} Despite this finding, in the absence of studies that assess the independent relation of assay levels to clinical outcome in these various patient categories, the clinically most relevant assay formats and their decision limits remain unknown.

In conclusion, several novel methods have been developed since the previous Round Robin for NTBI, including LPI and DCI assays and first attempts to specifically determine the NTBI iron-citrate isoforms. While NTBI and LPI values of various assays were well correlated and were reproducible, absolute values differed considerably. The assay(s) that best represent the clinically relevant species and are most relevant for diagnostic and therapeutic purposes cannot be determined from this study. Before NTBI and LPI assays can be introduced into clinical practice rigorous validation and standardization of assays, consensus on how to report the results, and clinical outcome studies to determine clinically relevant assay formats and their toxic thresholds are needed.

Acknowledgements
We would like to thank all Round Robin 2 NTBI laboratory participants and sample collectors for their contribution and the members of the advisory committee: prof. dr. Theo de Witte and prof. dr. Norbert Gattermann. Special thanks also to Siem Klaver for his support in logistics and Rian Roelofs and Erwin Wiegerinck for their technical assistance and valuable
discussions. This work was supported by an educational grant from Novartis Pharmacy B.V. Europe/The Netherlands to DS and LdS.

Authorship Contributions
Design: LdS, JH, DS; provision of patients, assembly of data: LdS, JH, IC, PE, EH, BW, GB, YF, MJ, BB, MM, VM, RO, RG, RH, DS; analyzing data: LdS, JH, LV, IC, PE, EH, GB, YF, JP, RO, RH, DS; manuscript drafting LdS and DS; manuscript editing LV, IC, GB, PE, EH, JH; final approval: all authors.
References

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<th>Age years</th>
<th>Hb mmol/L</th>
<th>MCV fl</th>
<th>CRP mg/L</th>
<th>LDH U/L</th>
<th>Iron μmol/L</th>
<th>Ferritin μg/L</th>
<th>TSAT immuno %</th>
<th>TSAT gel %</th>
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<td>6</td>
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<td>91 (82-96)</td>
<td>0 (0-0)</td>
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<td>37 (33-44)</td>
<td>1389 (948-2049)</td>
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<td>631 (570-786)</td>
<td>66 (35-89)</td>
<td>58 (31-95)</td>
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<td>181 (161-290)</td>
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<td>2633 (1379-11834)</td>
<td>94 (93-106)</td>
<td>100 (92-100)</td>
</tr>
<tr>
<td>Sickle Cell Disease</td>
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<td></td>
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<tr>
<td>9. Naive</td>
<td>6</td>
<td>34 (18-57)</td>
<td>5.2 (4.1-7.1)</td>
<td>87 (76-114)</td>
<td>0 (0-6)</td>
<td>437 (290-864)</td>
<td>19 (11-27)</td>
<td>164 (79-996)</td>
<td>37 (18-58)</td>
<td>37 (28-48)</td>
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<td>10. TD</td>
<td>6</td>
<td>23 (19-41)</td>
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<td>85 (81-90)</td>
<td>3 (0-30)</td>
<td>448 (231-649)</td>
<td>25 (7-38)</td>
<td>2091 (1451-5353)</td>
<td>62 (19-95)</td>
<td>51 (17-100)</td>
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</tbody>
</table>

**Table 1. Median (range) of patient characteristics by disease and treatment groups.** Median (range) are given for 5-7 measurements in each disease and treatment group. Naive, before start of any therapy; Depletion, during the depletion phase of phlebotomy treatment; Maintenance, during maintenance phase of phlebotomy. TD, transfusion-dependent; CH, receiving iron chelation therapy; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; Hb; Hemoglobin; MCV, Mean Corpuscular Volume; CRP, C-reactive protein; LDH, lactate dehydrogenase; Iron, serum iron; Ferritin, serum ferritin; TSAT immuno, transferrin saturation, transferrin measured immunochemically and iron with a colorimetric method, both on an automated analyzer; TSAT gel, transferrin saturation measured with gel electrophoresis; NA, not available; Hb and MCV in Thal patients were from the first sampling due to pooling afterwards. a, Hb (g/dL) = mmol/L x 1.6206; b, chelation consisted of Deferiprone (Ferriprox, DFP, n=2) and Deferasirox (Exjade, DFX, n=4); c, the disease groups have been given numbers as IDs; d, including 5 patients on iron chelation therapy that consisted of DFP (n=3) and DFX (=2); e, the hemolytic index (reflecting *in vivo* and *ex vivo* hemolysis) levels were within the reference range (<20 mg/dL) for all samples (data not shown). Patients with Sickle Cell Disease were of African descent (African-Dutch) and the patients with β-Thalassemia were all Italian-Caucasian (Italians). All other patients were Dutch-Caucasian.
<table>
<thead>
<tr>
<th>Assay ID</th>
<th>NTBI assays</th>
<th>LPI assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>N1</td>
<td>N2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>-------------</td>
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<tr>
<td>Disease/Total</td>
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<td>1.60 (.37)</td>
</tr>
</tbody>
</table>

**Hemochromatosis**

1. Naive 6 2.61 (.43) 0.42 (.19) 2.08 (.16) 3.25 (.73) 1.58 (.22) 0.21 (.11) 0.39 (.13) 0.10 (.06) 1.34 (.31)
2. Phlebotomy 6 0.36 (.30) -1.32 (.20) 0.66 (.13) 1.15 (.20) 1.36 (.15) -0.04 (.05) -0.09 (.14) 0.00 (.00) 0.03 (.04)
3. Maintenance 6 0.25 (.36) -1.52 (.15) 0.57 (.02) 0.91 (.35) 1.19 (.19) -0.07 (.03) 0.02 (.15) 0.00 (.00) 0.05 (.03)

**β-Thalassemia**

4. Major Naive 6 0.61 (.25) -1.19 (.17) 0.76 (.05) 1.68 (1.07) 1.08 (.36) -0.01 (.04) 0.08 (.09) 0.01 (.01) 0.17 (.05)
5. Major TD & CH 6 7.79 (.65) 0.38 (.27) 2.46 (.12) 4.00 (.67) 0.87 (.11) 0.43 (.12) 0.68 (.24) 0.54 (.15) 1.43 (.17)
6. Intermedia Naive 7 3.32 (.44) 0.04 (.17) 2.46 (.17) 3.74 (.25) 1.51 (.06) 0.28 (.10) 0.64 (.25) 0.52 (.06) 0.83 (.14)

**β-Thalassemia**

7. RARS/RCM-D-RS 6 0.14 (.16) -1.38 (.25) 0.61 (.03) 1.37 (.20) 1.11 (.05) -0.05 (.02) 0.04 (.17) 0.00 (.00) 0.02 (.03)
8. TD 5 2.97 (.81) 0.53 (.15) 2.33 (.43) 3.65 (.31) 0.60 (.04) 0.22 (.10) 0.62 (.23) 0.20 (.11) 0.66 (.29)

**Sickle Cell Disease**

9. Naive 6 -0.10 (.24) -2.01 (.15) 0.44 (.22) 0.49 (.12) 0.94 (.06) -0.06 (.04) -0.01 (.12) 0.00 (.00) 0.01 (.01)
10. TD & CH 6 -0.05 (.19) -1.12 (.32) 0.86 (.14) 1.69 (.20) 0.57 (.21) 0.00 (.04) -0.01 (.15) 0.00 (.00) 0.06 (.06)

Table 2. Mean (SD, in µmol/L) of the NTBI and LPI values by assay, disease and treatment. Assay ID: random numbering within assay group. Each mean was calculated as the outcome of the means of a total of 4 NTBI or LPI measurements (2 days, 2 measurements with exception of two methods, one which performed assays over 4 days, 1 measurement and one which performed 1 day, 2 measurements), according to the number of patients in each disease category (N=5-7). NTBI assays, measurement of total NTBI using chelators like NTA or desferrioxamine as scavenging agent; LPI assays, measurement of redox active fraction of NTBI using reducing agents like ascorbate or bleomycin to induce redox cycling and generate reactive oxygen species (ROS); NTBI isoform-specific, measurement of the iron-citrate fraction in the serum samples; DCI, direct chelatable iron; eLPI, enhanced LPI. Method N5 (DCI) has been omitted from this table since NTBI was only present in 8 % of the samples (N=5: 3 Thal major TD & CH, 1 Thal intermedia naive, 1 MDS TD);<sup>a,b</sup> for statistical calculations, results below the LLOD of 0.60 and 0.87 were included as 0.30 and 0.40, respectively;<sup>c</sup> results were recommended to be interpreted as follows: X <0.1 = negative, 0.1 ≥ X <0.2 = undetermined, X ≥0.2 = positive;<sup>d</sup> for statistical calculations, results above the ULOD of 2.2 were included as 2.4 µmol/L;<sup>e</sup> range of levels assessed in healthy individuals (23-61 yrs, 50% men; TSAT 16-56%): -2.29 to 0.35 µmol/L.
Legends to figures

Figure 1. Bland-Altman plots, showing differences in mean NTBI or LPI levels (µmol/L) between 2 methods versus the average of the mean values of these two methods. Solid lines represent the mean difference between the 2 methods. Thick dashed lines represent the 95% confidence interval. Thin dashed lines at 0 refer to no difference between methods. Dots represent the means of duplicates on day 2. Note that absolute values on both axis differ between plots.

Figure 2. Relationship between the assay concentrations and transferrin saturation (TSAT). Assay results are given for day 2 as duplicate measurements (circle and triangle).
Collection of samples

Serum samples (n=60) were collected from patients with 4 iron overload disorders in various treatment phases, making a total of 10 different patient and treatment groups. The 60 serum samples were split into two aliquots, coded, stored at -80°C and shipped for analysis on dry ice to the laboratories in May 2013. Laboratories performed duplicate measurements in a blinded fashion, on each aliquot of a serum sample on 2 different days, resulting in a total of 4 measurements for each sample. Some laboratories provided multiple assays. The participants reported their results within 5 weeks after receiving the samples.

Samples (n=18) from HH patients were collected at the Radboudumc between January 2004 and March 2012 after obtaining oral informed consent and approval of the local ethical committee. They were from 6 different patients with a homozygous p.Cys282Tyr mutation in the HFE-gene from 3 different stages of phlebotomy treatment: naive (n=6), during depletion (n=6) and during maintenance (n=6) therapy.

Samples from 19 different patients with Thal major and intermedia were collected at the University of Cagliari, Italy, between July 2011 and April 2012 and comprised residual sera from routine clinical assessment conforing to the code for proper secondary use of human tissue. In order to obtain sufficient volumes, samples from both Thal major and Thal intermedia patients consisted of 2-5 pooled aliquots of the same patient obtained within a period of 7 months.

All patients with Thal major were homozygous for the β°39 nonsense mutation. Thal major naive patients received transfusions but did not receive iron chelation therapy (n=6); Thal major TD & CH patients were transfusion-dependent (TD) and received iron chelation (CH) therapy (n=6), either Deferiprone (Ferriprox, DFP, n=2) or Deferasirox (Exjade, DFX, n=4). Two different genotypes of the β-globin gene were included in the Thal intermedia patients: homozygous for β°39 nonsense and compound heterozygous for β6 (-A)/β°39 nonsense. In these patients, the co-inheritance of α-thalassemia and the presence of the c.158C>T mutation in the Gγ-globin gene, associated with increased production of γ-globin chains in adult life, are the main determinants for the milder phenotype. Thal intermedia naive patients were untransfused and did not receive iron chelation therapy (n=7).

Samples (n=11) from patients with MDS were collected at Rijnstate hospital and Radboudumc, The Netherlands between January 2012 and May 2013 after obtaining oral informed consent and approval of the local ethical committee. Patients with MDS were diagnosed according to the WHO-2001 MDS classification system. Samples were collected from: i) transfusion-independent patients with MDS with refractory anemia with ringed sideroblasts (RARS, n=4) or refractory cytopenia with multilineage dysplasia with ringed sideroblasts (RCMD-RS, n=2); ii) transfusion-dependent patients with MDS RCMD (n=1), MDS RAEB-1 (n=2), MDS RAEB-2 (n=1), MDS therapy related (n=1), where transfusion-dependency was defined as serum ferritin ≥1000µg/L or a transfusion intensity of ≥1 red blood cell unit/8 weeks (MDS TD).

Samples (n=12) from patients with sickle cell disease (SCD) were collected, after obtaining oral informed consent and approval of the local ethical committee, in the Academic Medical Center (AMC) in Amsterdam, The Netherlands between March and May 2013. All SCD patients had homozygous sickle cell disease (HbSS) and were i) untransfused and without iron chelation therapy (SCD naive; n=6) and ii) transfusion-dependent, with serum ferritin ≥1000µg/L, including 5 patients on iron chelation therapy that consisted of DFP (n=3) and DFX (=2) (SCD TD; n=6). All patients were requested to abstain from iron chelation therapy for 24 hours before blood withdrawal.
**Laboratory analysis**

Hemoglobin and mean corpuscular volume (MCV) measurements were performed on automated hematology analyzers at the hospital of sample collection: MDS on a Sysmex XE-2100, Thalassemia samples on a Beckman Coulter LH750, Hemochromatosis samples on an ADVIA TM120, Sysmex XE-2100 or Sysmex XE-5000; SCD samples on a Sysmex XE-5000. C-reactive protein (CRP), lactate dehydrogenase (LDH), Hemolytic Index (HI) and all iron parameters were all determined at Radboudumc on an Architect c16000 (Abbott Diagnostics, Lake Forest, USA) or on an Immulite 2000 (Siemens healthcare Diagnostics, Deerfield, USA). Only LDH of the SCD and MDS patients (P800, Roche Diagnostics) and ferritin for SCD patients (E170 Roche Diagnostics) were measured on automated analyzers at the hospital of sample selection.

Reference ranges are from the clinical laboratory of Radboudumc.

Transferrin was measured using immunochemistry and total iron binding capacity (TIBC) was calculated as: TIBC (μmol/L) = 25.0 x transferrin (g/L). TSAT was calculated as: TSAT (%) = (serum iron (μmol/L) / TIBC (μmol/L)) x 100%. For all samples, TSAT was also determined by urea gel electrophoresis, separating the four forms of transferrin (apotransferrin, monoferric transferrin with iron bound to either the N-terminus or the C-terminus and holotransferrin) by polyacrylamide gel electrophoresis in the presence of 6M urea. This latter TSAT methodology is not affected by the presence of residual chelator or chelates.

**Statistical methods**

The study was designed to compare NTBI levels as well as the repeatability of the methods. With respect to the repeatability, the magnitude of variation that exists between samples and between measurements of the same sample relative to the total variation is of interest. Accordingly, we partitioned the total variance of each method into the following components: i) the between-sample variance and ii) the analytical variance. Our design allowed us to divide the latter into three subcomponents: the between-day variance, the between-duplicate variance and the residual analytical variance, i.e. the part of the analytical variance that cannot be attributed to the other two. A linear mixed model was used to estimate these variance components of each method separately. The dependent variable was the NTBI outcome, and the independent random variables were: sample (60 levels), day (two levels) and repeated measurement (two levels). The standard deviation (absolute error) and the percentage variance relative to the total variance are presented for each method separately.

For statistical calculations, for assays N3 and N4, results below the LLOD of 0.60 and 0.87 were included as 0.30 and 0.40 μmol/L, respectively; for assays L3 and L4, results above the ULOD of 2.2 were included as 2.4 μmol/L.
<table>
<thead>
<tr>
<th>Assay Group</th>
<th>Method ID</th>
<th>Assay subgroup</th>
<th>Laboratory¹</th>
<th>Chelator</th>
<th>Detection</th>
<th>Reported values (µmol/L)</th>
<th>References</th>
</tr>
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<td>N1</td>
<td>DCI</td>
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<td>CP851⁸</td>
<td>Fluorescence²</td>
<td>Full range + negatives</td>
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<td></td>
<td>N2</td>
<td>NTBI</td>
<td>2</td>
<td>NTA⁷</td>
<td>HPLC</td>
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<td>N3</td>
<td>NTBI</td>
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<td>LLOD &lt;0.60</td>
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<td>Ascorbate/DHR</td>
<td>Fluorescence⁶</td>
<td>Range ≥0.0-2.2</td>
<td>¹¹</td>
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**Supplemental table 1. Characteristics of methods for the quantification of serum NTBI and LPI.** Method ID, random numbering within assay group; the same number indicates the same laboratory: Laboratory 1, Institute of Pharmaceutical Science, King’s College London, UK; 2, Department of Haematology, University College London, UK; 3, Department of Pathology and Cell Biology, Columbia University Medical Center, New York, USA; 4, Department of Laboratory Medicine, Radboud University Medical Center, Nijmegen, the Netherlands; 5, Aferrix Ltd., Tel-Aviv, Israel; Fluorescent beads; using TGA (thioglycolic acid) and BPT (baptophenanthroline disulfonic acid); Fluoresceinated-deferrioxamine; TBA (thiobarbituric acid) to detect malondialdehyde as an iron-bleomycin induced degradation product of DNA; Oxidation-sensitive probe (Dihydrorhodamine (DHR)); concentration used was 80 mM; a hexadendate pyridine chelator; 0.1 mM NTA; tests are commercially available, FeROS™ LPI¹⁰a and FeROS™ eLPI¹⁰b, respectively, these products are CE marked for in-vitro diagnostic use (IVD). *measurements were on four different days instead of twice on two different days, LLOD was based on three times the SD of a blank water sample; LLOD was based on three times the SD of a blank serum sample. NTBI, non-transferrin-bound iron; LPI, labile plasma iron; DCI, directly chelatable iron; NTA, nitrilotriacetic acid; DFO, desferrioxamine; e-LPI, enhanced-labile plasma iron; ICP-MS, inductively coupled plasma mass spectrometry.
<table>
<thead>
<tr>
<th>Assay</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N6</th>
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<th>L2</th>
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</table>

Supplemental Table 2. Statistical ordering of the disease/treatment groups per assay, using a multi-linear mixed model. Numbers represent the IDs of the disease and treatment groups as given in Tables 1 and 2. The brackets indicate non-statistically significant differences according to the Tukey test. Numbers refer to “disease groups” as given in table 2, i.e. 10 combinations of disease and treatment. The linear mixed model was used to study the differences between the combinations of disease and treatment for each method separately. The independent variable was the NTBI measured with a specific method. The independent fixed variable was “disease” (10 levels) and the intercept of each serum sample was treated as a random variable. The differences between disease/treatment groups are based on estimated mean NTBI and the appropriate standard errors of each combination of disease and treatment of each method separately.
<table>
<thead>
<tr>
<th>Method</th>
<th>Proportion samples with NTBI or LPI (%) stratified by TSAT</th>
</tr>
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<td>47.1 (30.3-63.8)</td>
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<tr>
<td>L4</td>
<td>76.5 (62.2-90.7)</td>
</tr>
</tbody>
</table>

**Supplemental table 3.** Proportion of samples (5-95% confidence intervals (CI)) with NTBI or LPI (%) > 0, stratified by TSAT. Proportions are calculated based on the first measurement of day 2.
Supplemental Figure 1. Correlation plots between assays, using the mean of the duplicate measurements on day two. A, B and C match the upper left, lower left and lower right quadrant of table 4 of the article, respectively. The Spearman correlation coefficients of each plotted combination of two assays can be found in the respective cell in table 4. Absolute values on both axis are expressed in µmol/L and differ between plots.
References