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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

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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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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 ten worldwide leading assays (6 for non-transferrin-bound iron and 4 for labile plasma iron) as part of an international inter-laboratory study. Serum samples from 60 patients with four different iron-overload disorders in various treatment phases were coded and sent in duplicate for analysis to five 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 correlated 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 of the presence of forms of circulating non-transferrin-bound iron. 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 heme and ferritin in cells. However, the iron binding capacity of the iron transport protein transferrin can be exceeded when a substantial amount of iron enters the circulation due to excessive iron absorption from the diet or release of iron from cell stores. In these conditions non-transferrin-bound iron (NTBI; circulating iron not bound to transferrin, ferritin or heme) appears in the plasma.[1-5] Plasma NTBI apparently comprises several subspecies, which may be classified by their chemical composition, chemical reactivity or susceptibility to chelation.[6-15] The chemical composition of NTBI is heterogeneous and it is thought that there are several circulating isoforms, that is Fe(III) bound to albumin and citrate and potentially to acetate, malate and phosphate.[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.[8,16] The fraction of plasma NTBI that is redox active and can be chelated is designated labile plasma iron (LPI).[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.[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 major and intermedia), myelodysplastic syndromes (MDS) and sickle-cell disease (SCD) after transfusion.[2,3,5] To prevent iron-induced tissue damage, impending iron toxicity must be detected 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 such as inflammation and liver disease and are not, therefore, exclusively indicative of toxic parenchymal iron overload.[17] Moreover, with the introduction of T2*-weighted magnetic resonance imaging for the assessment of tissue iron overload,[18] it became 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 patients with thalassemia major suggest that NTBI may be an important early indicator of extra-hepatic iron toxicity.[19] Studies in patients with various iron-loading disorders have shown reductions in NTBI and LPI upon phlebotomy and chelation therapy and that these reductions are associated with an improved prognosis.[2,3,20-23] The results of NTBI and LPI assays are, therefore, promising as therapeutic targets and for the evaluation of iron overload and the efficacy of and compliance with iron-lowering therapies.[5,13,24]

Due to the complexity and potential clinical importance of NTBI, several assays have been developed for its detection.[6,10-12,15,25-29] In our previous round robin 1 we found that NTBI values of patients with HFE-related hemochromatosis differed considerably depending on which of various assays was used.[30] We concluded that NTBI assays were insufficiently standardized and the most pertinent assay for clinical applications was uncertain. Since that round robin 1 for NTBI, novel assays have been published and made available for use.[12,15,29] Therefore, and as a stepping stone in the path to defining the clinical utility of these assays, the aims of our study were to update round robin 1 and to 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 (those with HH, thalassemia, MDS, and SCD) undergoing various treatments (phlebotomy, iron chelation, red blood cell transfusion). More specifically, in these populations of patients, we aimed to: (i) establish correlations between assays, (ii) establish levels of reproducibility of each of the assays, (iii) assess levels of the NTBI fraction consisting of iron citrate, and (iv) correlate assays with other established parameters of iron overload (e.g. TSAT and serum ferritin).

Methods

Study design and participants

We compared ten different assays (5 NTBI, 1 NTBI isoform-specific and 4 LPI) performed in five different laboratories worldwide. Serum samples (n=60) were collected from patients with four iron overload disorders in different treatment phases (transfusion-dependent; receiving iron chelation) making a total of ten different patient and treatment groups. Samples were collected with approval from local Institutional Review Boards and in conformity with the code for proper secondary use of human tissues. More detailed information on the collection of samples and laboratory analyses are given in the *Online Supplementary Information*.

Non-transferrin-bound iron and labile plasma iron assays

The NTBI/LPI assays were divided into three different groups: (i) five NTBI assays (N1-N5); (ii) one NTBI isoform-specific assay (N6); and (iii) four LPI assays (L1-L4) (*Online Supplementary Table S1*). The test principles of the NTBI assays can be divided into two subgroups. The first subgroup (N2-4) consists of a chelation-ultrafiltration-detection approach based on the prior mobilization of serum NTBI by weak iron-mobilizing chelators such as nitrilotriacetate at 80 mM. The chelated NTBI is separated from transferrin-bound iron by ultrafiltration and detected by colorimetry or high performance liquid chromatography.[26-28] The second subgroup of NTBI assays (N1 and N5) comprises the measurement of directly chelatable iron. 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.[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 high performance liquid chromatography to separate the individual components of NTBI by mass, followed by quantification of iron by inductively coupled-plasma mass spectrometry (LC-ICP-MS).[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 are generated which degrade the deoxyribose moieties of the DNA in a site-specific reaction. These degradation products bind to thiobarbituric acid to

give a pink chromogen for quantification.[25] The assays L2, L3 and L4 used a reducing agent (ascorbate) and an oxidizing agent (atmospheric O_2) to generate reactive oxygen species from endogenous oxidants and labile catalytic iron. Reactive oxygen species were detected by an oxidation-sensitive probe (dihydrorhodamine). 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.[12,29] For the extraction of iron from NTBI, assay L4 used 0.1 mM nitrilotriacetate to capture the mobilizer-dependent form of LPI (enhanced LPI).[11, 29]

The statistical analysis is described in detail in the *Online Supplementary Information*.

Results

Patients' characteristics

The characteristics of the patients, divided by disease and treatment group, are shown in Table 1. Serum ferritin levels were highest in patients with transfusion-dependent thalassemia major, MDS or SCD, in thalassemia major patients receiving iron chelation therapy and in HH patients at presentation. The ferritin levels in HH patients decreased after phlebotomy. TSAT levels were similar for the two different TSAT methodologies used and were highest in thalassemia major patients who were transfusion-dependent or receiving iron chelation (median 100%). The median TSAT was also very high (>94%) in patients with HH or thalassemia intermedia at presentation, transfusion-dependent MDS and less increased (>51%) in patients with thalassemia major at presentation, in the depletion phase of phlebotomies in HH patients, in MDS subtypes refractory anemia with ringed sideroblasts/refractory cytopenia with multilineage dysplasia with ringed sideroblasts and in transfusion-dependent SCD. The median TSAT was within the reference range (<45%) for most SCD patients at presentation 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,

Table 1. Patients' characteristics by disease and treatment groups. Median (range) are given for five to seven measurements in each disease and treatment group.

Disease[c]	N	Age years		Hb mmol/L[a]		MCV fL		CRP mg/L		LDH[e] U/L	Iron μmol/L	Ferritin μg/L	TSAT immuno %	TSAT gel %
Hemochromatosis														
1. Naïve	6	58	(42-66)	9.5	(8.4-10.0)	91	(82-96)	0	(0-0)	157 (128-238)	37 (33-44)	1389 (948-2049)	97 (76-100)	100 (80-100)
2. Depletion	6	59	(42-66)	8.5	(7.9-9.0)	93	(85-104)	0	(0-0)	121 (105-140)	31 (18-39)	631 (570-786)	66 (35-89)	58 (31-95)
3. Maintenance	6	59	(43-67)	9.2	(8.3-9.6)	90	(82-97)	0	(0-0)	126 (112-144)	23 (10-36)	58 (30-166)	44 (21-95)	43 (24-100)
β-Thalassemia														
4. Major Naïve	6	1	(0.5-2)	5.7	(4.6-6.6)	81	(73-84)	0	(0-5)	252 (233-478)	24 (17-29)	912 (639-2060)	52 (43-94)	52 (46-100)
5. Major TD & CH[b]	6	31	(28-35)	6.0	(5.4-6.3)	84	(82-86)	0	(0-0)	127 (91-141)	38 (17-46)	2507 (623-5451)	100 (45-103)	100 (47-100)
6. Intermedia Naïve	7	39	(22-60)	5.5	(4.7-6.3)	85	(64-94)	0	(0-6)	228 (150-550)	35 (16-42)	487 (306-940)	95 (42-103)	100 (48-100)
MDS														
7. RARS/RCMD-RS	6	69	(26-77)	6.7	(5.4-7.4)	97	(79-99)	0	(0-0)	190 (105-292)	35 (17-39)	474 (289-826)	71 (31-90)	52 (31-88)
8. TD	5	71	(68-85)	4.6	(4.0-5.6)	92	(87-97)	20	(0-275)	181 (161-290)	30 (19-45)	2633 (1379-11834)	94 (93-106)	100 (92-100)
Sickle cell disease														
9. Naïve	6	34	(18-57)	5.2	(4.1-7.1)	87	(76-114)	0	(0-6)	437 (290-864)	19 (11-27)	164 (79-996)	37 (18-58)	37 (28-48)
10. TD[d]	6	23	(19-41)	5.9	(4.2-6.5)	85	(81-90)	3	(0-30)	448 (231-649)	25 (7-38)	2091 (1451-5353)	62 (19-95)	51 (17-100)
Reference range														
Male	–	–		8.5-11.0		80-100		<10		<250	14-35	25-250	<45	<45
Female	–	–		7.5-10.0		80-100		<10		<250	10-25	20-250	<45	<45

Naïve: 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 thalassemia 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 identities; [d]: including five 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 SCD were of African descent (African-Dutch) and the patients with β-thalassemia were all Italian-Caucasian (Italians). All other patients were Dutch-Caucasian.

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 directly chelatable iron but levels were 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 from further analysis.

As expected, levels of assay L4, which measures the forms of LPI that are detectable in the presence of nitrilotri-acetate (designated "enhanced LPI"), were higher than those obtained by assay L3 that uses a methodology that is identical to L4 except that nitrilotriacetate is not added.

Assays differed widely in the use of predefined detection limits (*Online Supplementary Table S1*). Four out of ten methods studied 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 two of these assays, L3 and L4, an upper limit of detection (LOD) of 2.2 μmol/L was also employed since the assay is non-linear above this concentration. Three assays (N3, N4, and N6)

Table 2. Mean (SD, in μmol/L) of the NTBI and LPI values by assay, disease and treatment.

Assay ID Assay subgroup	N	NTBI assays					LPI assays			
		N1 DCI	N2[e] NTBI	N3[a] NTBI	N4[b] NTBI	N6 Isoform	L1 LPI	L2 LPI	L3[c,d] LPI	L4[c,d] eLPI
Disease/Total	60	1.60 (0.37)	-0.73 (0.20)	1.32 (0.14)	2.19 (0.64)	1.10 (0.15)	0.09 (0.07)	0.24 (0.17)	0.14 (0.04)	0.46 (0.11)
Hemochromatosis										
1. Naïve	6	2.61 (0.43)	0.42 (0.19)	2.08 (0.16)	3.25 (0.73)	1.58 (0.22)	0.21 (0.11)	0.39 (0.13)	0.10 (0.06)	1.34 (0.31)
2. Phlebotomy	6	0.36 (0.30)	-1.32 (0.20)	0.66 (0.13)	1.15 (0.20)	1.36 (0.15)	-0.04 (0.05)	-0.09 (0.14)	0.00 (0.00)	0.03 (0.04)
3. Maintenance	6	0.25 (0.26)	-1.52 (0.15)	0.57 (0.02)	0.91 (0.35)	1.19 (0.19)	-0.07 (0.03)	0.02 (0.15)	0.00 (0.00)	0.05 (0.03)
β-Thalassemia										
4. Major Naïve	6	0.61 (0.25)	-1.19 (0.17)	0.76 (0.05)	1.68 (1.07)	1.08 (0.36)	-0.01 (0.04)	0.08 (0.09)	0.01 (0.01)	0.17 (0.05)
5. Major TD & CH	6	5.79 (0.65)	0.38 (0.27)	2.46 (0.12)	4.00 (0.67)	0.87 (0.11)	0.43 (0.12)	0.68 (0.24)	0.54 (0.15)	1.43 (0.17)
6. Intermedia Naïve	7	3.32 (0.44)	0.04 (0.17)	2.46 (0.17)	3.74 (2.25)	1.51 (0.06)	0.28 (0.10)	0.64 (0.25)	0.52 (0.06)	0.83 (0.14)
MDS										
7. RARS/RCMD-RS	6	0.14 (0.16)	-1.38 (0.25)	0.61 (0.03)	1.37 (0.20)	1.11 (0.05)	-0.05 (0.02)	0.04 (0.17)	0.00 (0.00)	0.02 (0.03)
8. TD	5	2.97 (0.81)	0.53 (0.15)	2.33 (0.43)	3.65 (0.31)	0.60 (0.04)	0.22 (0.10)	0.62 (0.23)	0.20 (0.11)	0.66 (0.29)
Sickle cell disease										
9. Naïve	6	-0.10 (0.24)	-2.01 (0.15)	0.44 (0.22)	0.49 (0.12)	0.94 (0.06)	-0.06 (0.04)	-0.01 (0.12)	0.00 (0.00)	0.01 (0.01)
10. TD & CH	6	-0.05 (0.19)	-1.12 (0.32)	0.86 (0.14)	1.69 (0.20)	0.57 (0.21)	0.00 (0.04)	-0.01 (0.15)	0.00 (0.00)	0.06 (0.06)

Assay ID: random numbering within assay group. Each mean was calculated as the outcome of the means of a total of four NTBI or LPI measurements (2 days, 2 measurements with exception of 2 methods, 1 which performed assays over 4 days, 1 measurement and 1 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 such as nitrilotriacetate or desferrioxamine as scavenging agent; LPI assays: measurement of redox active fraction of NTBI using reducing agents such as ascorbate or bleomycin to induce redox cycling and generate reactive oxygen species; 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 thalassemia major, transfusion-dependent or receiving iron chelation therapy, 1 untreated thalassemia intermedia, 1 transfusion-dependent MDS); [a,b]: for statistical calculations, results below the LLOD of 0.60 and 0.87 were included as 0.30 and 0.40, respectively; [c]: results were recommended to be interpreted as follows: X <0.1 =negative, 0.1 ≥ X <0.2 = undetermined, X ≥0.2 = positive; [d]: for statistical calculations, results above the upper LOD of 2.2 were included as 2.4 μmol/L; [e]: range of levels assessed in healthy individuals (23-61 years, 50% men; TSAT 16-56%): -2.29 to 0.35 μmol/L.

Table 3. The between-sample and within-sample variation of the assay concentrations by method.

Method	Total	SD (μmol/L)				Variance (%)			
		Between-sample	Within-sample		Residual	Between-sample	Within-sample		Residual
			Within-day	Between-day			Within-day	Between-day	
N1	3.20	3.15	0.00	0.15	0.51	97.2	0.0	0.2	2.6
N2	1.23	1.19	0.00	0.18	0.21	94.8	0.0	2.2	3.0
N3	1.18	1.15	NA	0.06	0.25	95.3	NA	0.2	4.5
N4	2.07	1.70	0.07	0.00	1.19	67.1	0.1	0.0	32.8
N6	1.08	1.05	0.00	0.00	0.26	94.3	0.0	0.0	5.8
L1	0.26	0.24	0.00	0.03	0.08	87.7	0.0	1.3	11.0
L2	0.51	0.47	0.00	0.00	0.21	83.5	0.0	0.0	16.5
L3	0.40	0.38	0.00	0.00	0.10	94.0	0.0	0.0	6.0
L4	0.69	0.67	0.00	0.00	0.18	93.5	0.0	0.0	6.5

SD: standard deviation, absolute error; between-sample SD: segment due to variation between samples; within-sample within-day: segment due to variation in repeated measurements on the same day; within-sample between-day: segment due to variation in repeated measurements on two different days. NA: not available, measurements were on four different days instead of twice on two different days. For N6 only a duplicate measurement on day 2 is available because of iron contamination issues on day 1.

measured positive values only. Two of them (N3 and N4) applied a lower LOD of 0.60 μmol/L and 0.87 μmol/L with reported results below the lower LOD in 42% and 32% of the samples, respectively.

Reproducibility

Assays gave a large 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 groups of patients (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.

Table 4. Spearman correlation coefficients between the assays, using the mean of the duplicate measurements on day 2.

	N1	N2	N3	N4	N6	L1	L2	L3
N2	0.60	–						
N3	0.57	0.88	–					
N4	0.57	0.85	0.86	–	–			
N6	-0.06	-0.06	-0.06	-0.11				
L1	0.50	0.89	0.85	0.83	0.05	–		
L2	0.59	0.54	0.48	0.44	0.05	0.54	–	
L3	0.75	0.73	0.69	0.62	0.03	0.72	0.74	–
L4	0.62	0.71	0.63	0.58	0.13	0.67	0.57	0.77

Bland-Altman plots of the bold numbers are presented in Figure 1. Correlation plots of all cells are provided in Online Supplementary Figure S1.
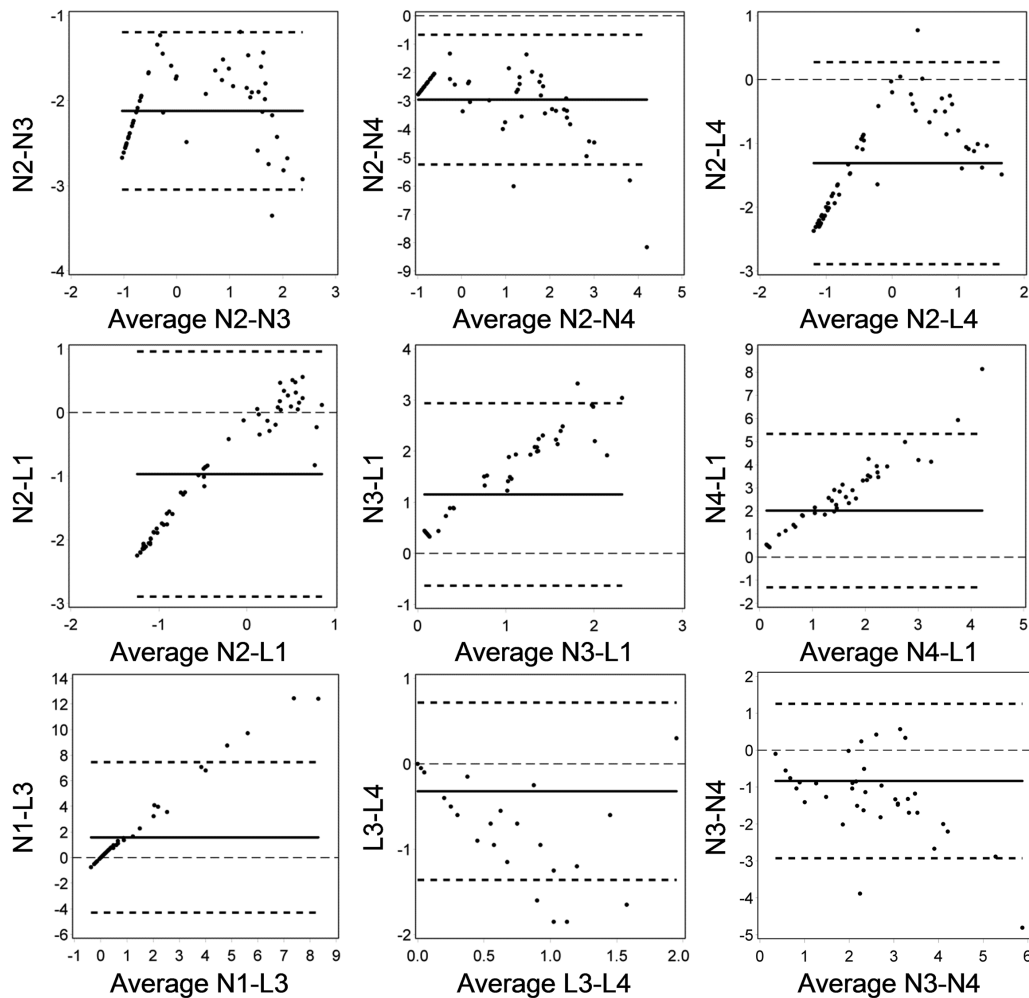
Figure 1. Bland-Altman plots showing differences in mean NTBI or LPI levels (μmol/L) between two methods *versus* the average of the mean values of these two methods. Solid lines represent the mean difference between the two methods. Thick dashed lines represent the 95% confidence interval. Thin dashed lines at 0 refer to no difference between methods. Dots represent the means of duplicates on day 2. Note that absolute values on both axes differ between plots.

Comparison between assays

Overall, assays correlated well and correlations were highest within the same group of NTBI or LPI assays (Table 4, *Online Supplementary Figure S1*). The NTBI and LPI assays with the highest mutual Spearman 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 than with the LPI assays. In contrast, NTBI assay N1 showed a better Spearman correlation with LPI assays than with NTBI assays (Table 4).

Figure 1 shows the Bland–Altman plots of a selection of nine 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, there is a straight line pattern (e.g. in the N2-L1 plot); and (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 ten assays in the ten 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) (*Online Supplementary Table S2*). Overall, assays were best in discriminating LPI and NTBI from patients with highest TSAT levels (thalassemia major patients who are transfusion-dependent or receiving iron chelation therapy, thalassemia intermedia patients, transfusion-dependent MDS patients and untreated HH patients) from those of the diseases with lower TSAT (untreated and transfusion-dependent SCD, HH maintenance; Table 2 and *Online Supplementary Table S2*).

Comparison of assay results with transferrin saturation and ferritin levels

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 exceed 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 in which 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% (*Online Supplementary Table S3*).

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 four different iron-over-

load diseases in various treatment phases. Nine out of the ten assays reported an assay value above zero in ≥30% of the samples. These nine 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 con-
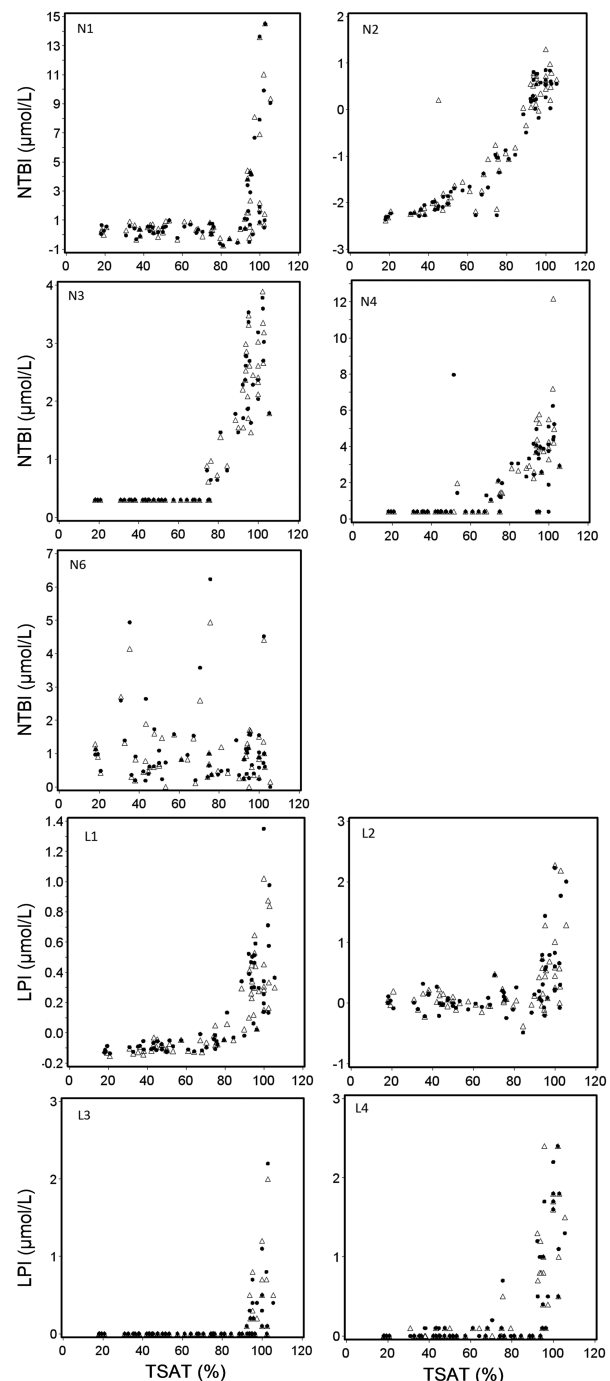
Figure 2. Relationship between the assay concentrations and transferrin saturation (TSAT). Assay results are given for day 2 as duplicate measurements (circles and triangles).

siderably 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 directly chelatable iron, NTBI-iron citrate, LPI and enhanced LPI.[3,11] In addition, minor variations in analytical procedures between methods aimed 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 nitrilotriacetate-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-nitrilotriacetate complexes do not pass the membrane, leading to underestimation.[31] The LPI assays also differ with regards to 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 nitrilotriacetate-filtration assays (N2-N4), but not in others.[11]

Furthermore, iron contamination may also be a source of variation in absolute assay concentrations.[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 nitrilotriacetate to vacant binding sites on transferrin.[27,31,33] Thus samples containing unsaturated transferrin may have lower NTBI values compared to standards in which 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 nitrilotriacetate.[34] Still, others have reported that the addition of cobalt (III) may result in substantial iron-contamination that could account for a rise in NTBI even in normal individuals.[32] Finally, with regards to the iron chelator nitrilotriacetate, applied in three NTBI assays, the use of 80 mM was shown by some authors to remove 1-8% of the iron bound to transferrin in the case of elevated TSAT, thus leading to overestimation of NTBI measurements.[27,31] By contrast, at physiological concentrations of serum iron in control subjects others reported no mobilization of iron from transferrin under these conditions.[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 Fe(III)-citrate complexes of NTBI was developed and included as N6 in the current round robin.[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 for 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, the 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 best to report assays before their introduction in clinical use. Four out of ten 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 below the lower LOD 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.[2,3,35,36] Altogether, our findings corroborate previous reports in HH and thalassemia patients that TSAT levels of around 70% can be considered as a threshold for the presence of potentially toxic iron species.[2,3] Nonetheless, because a single measurement only reflects the labile iron species present during the previous 24-48 hours, repeated measurements are imperative to assess a potential risk of impending NTBI toxicity.[18]

The majority of the assays in the current round robin have given useful insights into the efficacy of iron chelation in transfusional siderotic patients and of phlebotomy in patients with HH.[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 categories of patients, 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 directly chelatable iron 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 could not 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.

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