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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Online supplemental information

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 conforming 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). [1, 2]

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.[3] 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 a 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. [4] 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.

Assay Group	Method ID	Assay subgroup	Laboratory[1]	Chelator	Detection	Reported values (μmol/L)	References
NTBI	N1	DCI	1	CP851[8]	Fluorescence[2]	Full range + negatives	[5]
	N2	NTBI	2	NTA[7]	HPLC	Full range + negatives	[6] [7]
	N3*	NTBI	3	NTA[7]	Colorimetric[3]	LLOD <0.60	[7]
	N4[+]	NTBI	4	NTA[7]	Colorimetric[3]	LLOD <0.87	[7]
	N5	DCI	5	DFO	Fluorescence[4]	Range ≥0.0	[8]
NTBI Isoform-specific	N6	NTBI iron-citrate	1	Liquid Chromatography	ICP-MS	Full range	[9]
	Method	**Assay subgroup**	**Laboratory**	**Agents**	**Detection**	**Reported values**	**Reference**
LPI	L1	LPI	2	Ascorbate/Bleomycin/DNA	Colorimetric[5]	Full range + negatives	[10]
	L2	LPI	2	Ascorbate/DHR	Fluorescence[6]	Full range + negatives	[11]
	L3	LPI[10a]	5	Ascorbate/DHR	Fluorescence[6]	Range ≥0.0-2.2	[11]
	L4	eLPI[10b]	5	NTA[9]/ascorbate/DHR	Fluorescence[6]	Range ≥0.0-2.2	[12]

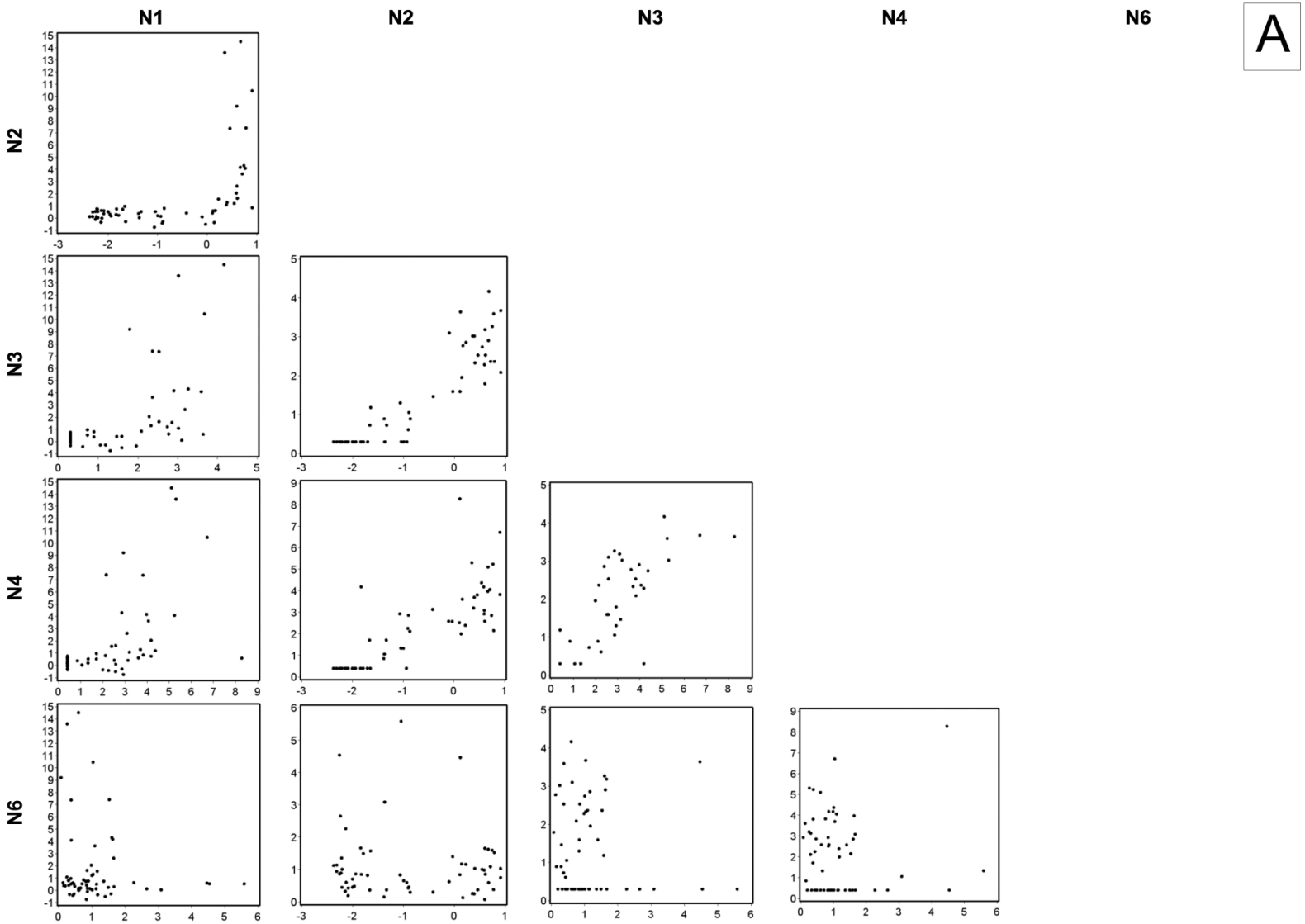
Supplemental table 1. Characteristics of methods for the quantification of serum NTBI and LPI. Method ID, random numbering within assay group; [1] the same number indicates the same laboratory; Laboratory 1, Institute of Pharmaceutical Science, King's College London, UK; 2, Department of Haematology, 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; [2]Fluorescent beads; [3] using TGA (thioglycolic acid) and BPT (baptophenanthroline disulfonic acid); [4] Fluoresceinated-deferrioxamine; [5] TBA (thiobarbituric acid) to detect malondialdehyde as an iron-bleomycin induced degradation product of DNA; [6] Oxidation-sensitive probe (Dihydrorhodamine (DHR)); [7]concentration used was 80 mM; [8]a hexadendate pyridine chelator; [9] 0.1 mM NTA; [10], tests are commercially available, FeROS™ LPI[10a] and FeROS™ eLPI[10b], 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.

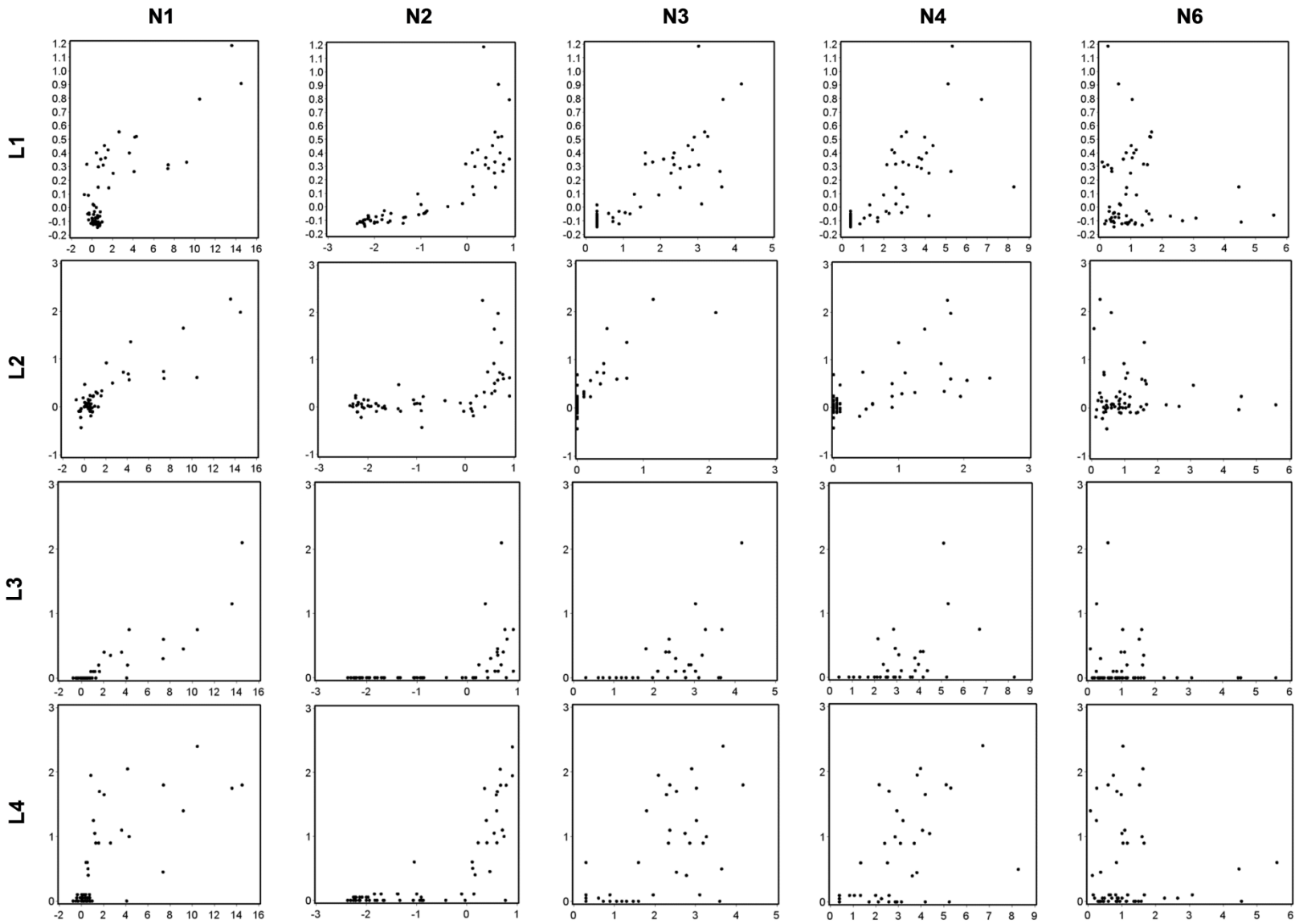
Assay	N1	N2	N3	N4	N6	L1	L2	L3	L4
Overall significance (P)	<0.0001	<0.0001	<0.0001	<0.0001	0.2678	<0.0001	<0.0001	<0.0001	<0.0001
Disease	5 6 8 1 4 2 3 7 10 9	8 1 5 6 10 4 2 7 3 9	6 5 8 1 10 4 2 7 3 9	5 6 8 1 10 4 7 2 3 9	1 6 2 3 7 4 9 5 8 10	5 6 8 1 10 4 2 7 9 3	5 6 8 1 4 7 3 10 9 2	5 6 8 1 4 3 7 2 9 10	5 1 6 8 4 10 3 2 7 9

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.

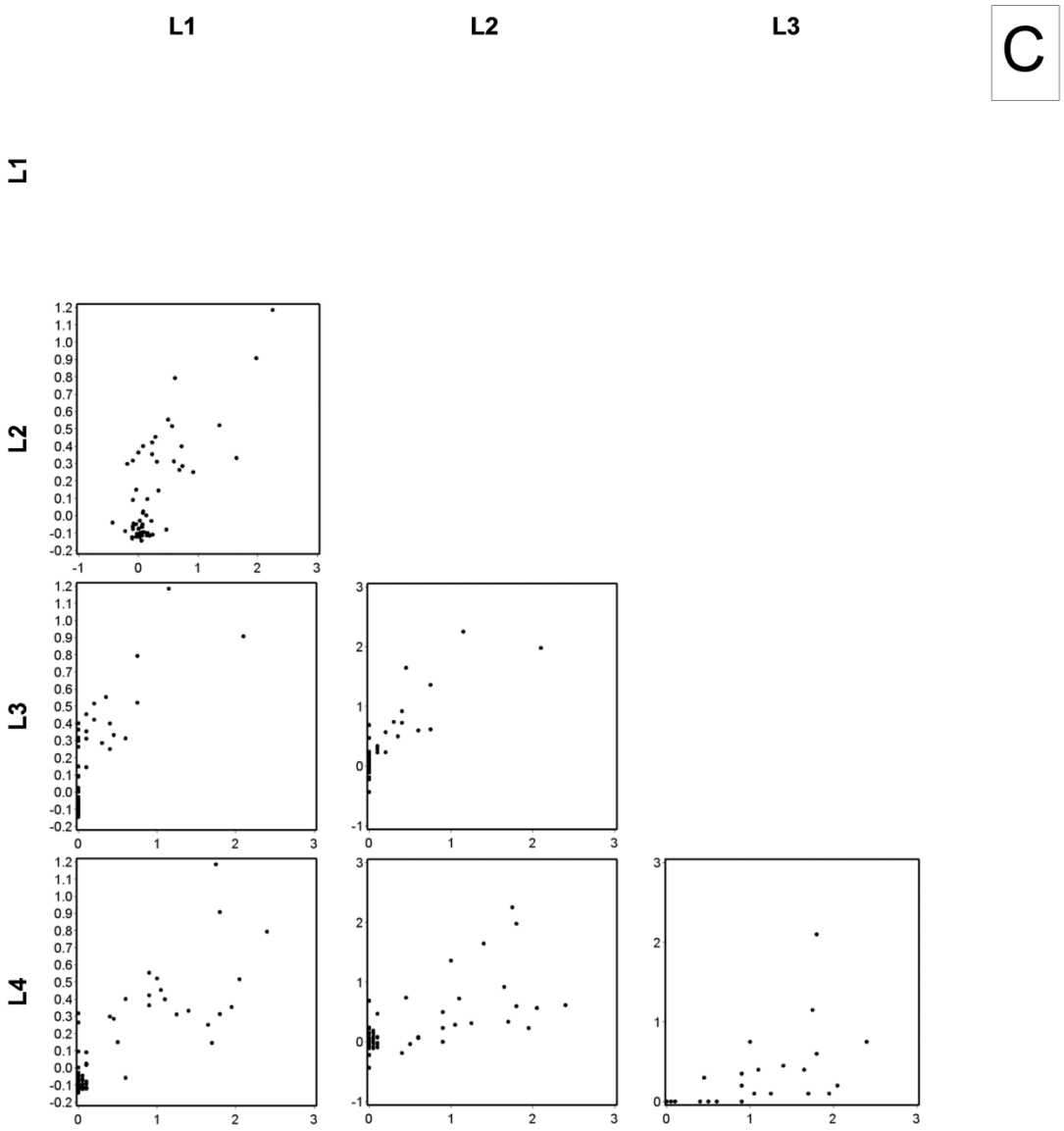
Method	Proportion samples with NTBI or LPI (%) stratified by TSAT							
ID	**>70%**	**(95% CI)**	**>80%**	**(95% CI)**	**>90%**	**(95% CI)**	**>95%**	**(95% CI)**
N1	82.4	(69.5-95.2)	85.2	(71.8-98.6)	95.7	(87.3-100)	100	(100-100)
N2	64.7	(48.6-80.8)	81.5	(66.8-96.1)	95.7	(87.3-100)	93.3	(80.7-100)
N3	100	(100-100)	100	(100-100)	100	(100-100)	100	(100-100)
N4	97.1	(91.4-100)	100	(100-100)	100	(100-100)	100	(100-100)
N5	14.7	(2.8-26.6)	18.5	(3.9-33.2)	21.7	(4.9-38.6)	33.3	(9.5-57.2)
N6	100	(100-100)	100	(100-100)	100	(100-100)	100	(100-100)
L1	73.5	(58.7-88.4)	92.6	(82.7-100)	100	(100-100)	100	(100-100)
L2	79.4	(65.8-93.0)	81.5	(66.8-96.1)	87.0	(73.2-100)	93.3	(80.7-100)
L3	47.1	(30.3-63.8)	59.3	(40.7-77.8)	69.7	(50.8-88.4)	86.7	(69.5-100)
L4	76.5	(62.2-90.7)	81.5	(66.8-96.1)	95.7	(87.3-100)	100	(100-100)

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.


N1
N2
N3
N4
N6
A



B



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.

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