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The relation between chemically measured total iron-binding capacity concentrations and immunologically measured transferrin concentrations in human serum

Raymond Gambino,[1]* Edouard Desvarieux,[1] Michael Orth,[2] Holly Matan,[3] Tomy Ackattupathil,[1] Elenore Lijoi,[1] Cathy Wimmer,[1] John Bower,[3] and Elaine Gunter[4]

We sought to determine if serum total iron-binding capacity (TIBC) is equivalent to serum transferrin (TRF) so that a low-cost colorimetric chemical assay for unsaturated iron-binding capacity (UIBC) could be substituted for a high-cost immunologic assay for TRF. Our study design included independent and blinded measurements of UIBC, serum iron, and TRF concentrations in human serum samples. Data from five independent correlation studies carried out at three different Quest Diagnostics laboratories were combined into one data set containing 570 paired results for TIBC and TRF. r^2 was 0.941 when three outliers were eliminated from the 570-sample data set. Scatter about the regression line was fully accounted for by the CVs for the TIBC and TRF assays. When each test is measured precisely and without bias, the ratio of TIBC (μmol/L) to TRF (g/L) in SI units is close to the theoretically expected value of 25.0.

Theoretically, 2 mol of iron (molecular mass 55.84 Da) bind to 1 mol of serum transferrin (TRF) (80 000 Da).[5] Chemical theory, therefore, predicts that 25.0 μmol of iron will saturate the binding sites of 1 g of TRF *[1, 2]*. If the binding of iron to TRF is uncomplicated, then a high degree of correlation between the two measurements is likely and the expected ratio of total iron-binding capacity (TIBC) in μmol/L to TRF in g/L should be 25.0 (i.e., TIBC μmol/L = 25.0 × TRF g/L). Tsung et al. report that TIBC and TRF are indeed equivalent *[3]*.

The relation between the two measurements, however, could vary a great deal if iron binds to other proteins to any significant degree, or if the binding of iron to TRF is altered as iron concentrations change, or if either assay has poor precision or bias, or both.

Several articles suggest that, contrary to theory, the relation between TIBC and TRF is not fixed, especially when results are outside the reference range *[4, 5]*. The reported mean ratio between TIBC and TRF has ranged from a low of 11.3 to a high of 26.1 *[6, 7]*.

We undertook this multilaboratory study of the relation between TIBC and TRF to investigate these discrepancies.

Design of Experiments

ANALYTICAL METHODS

TIBC was calculated from the sum of measured unsaturated iron-binding capacity (UIBC) and measured serum iron (i.e., TIBC = UIBC + serum iron). At all three laboratories, UIBC and serum iron were measured colorimetrically on either Hitachi 736–50 (Boehringher Mannheim Corp.) or Olympus AU5200 (Olympus America) high-throughput chemistry analyzers with reagents and aqueous or bovine-based calibrators from Diagnostic Chemicals Limited (DCL). The DCL assay includes Ferene® to form an iron–ferritin complex with maximum absorption at 593 nm and a molar absorptivity of 35 500.

The UIBC assay is essentially identical on each of these high-throughput chemistry analyzers. A precisely known amount of iron in solution is added to diluted serum. The added iron binds to previously unsaturated binding sites on TRF. After a short time—<5 min—the amount of iron that did not bind to TRF is measured colorimetrically.

Quest Diagnostics Inc., [1] 1 Malcolm Ave., Teterboro, NJ 07608-1070; [2] Wallingford, CT 08492; and [3] Horsham, PA 19044.
[4] Centers for Disease Control and Prevention, Atlanta, GA 30341.
*Author for correspondence. Fax 201-393-5903; e-mail Doclab@aol.com.
[5] Nonstandard abbreviations: TRF, transferrin; TIBC, total iron-binding capacity; and UIBC, unsaturated iron-binding capacity.
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UIBC is defined as the difference in the color produced by the original amount of added iron (highest color) and the color produced by the residual iron in solution that did not bind to TRF (less color).

TRF was measured immunologically on either the Behring Nephelometer II (Behring Diagnostics) or the Beckman Array® 360-CE (Beckman Instruments), or both, with respective calibrators from Behring or Beckman. The calibrators were standardized to the IFCC's preparation for plasma proteins Clinical Reference Material lot 470, which was certified by the Bureau of Reference of the European Community and designated the Reference Preparation for Proteins in Human Serum by the College of American Pathologists. This new calibrator yields TRF concentrations that are 13% lower than those obtained with the older calibrator. Table 1 specifies the nephelometer used and in which laboratory. The two nephelometers differ in how they measure the protein concentrations. The Beckman Array monitors the rate at which scattered light changes. The Behring nephelometer measures the amount of light scattered at a defined end point.

PRECISION STUDIES

Two independent precision studies were conducted: one in March 1996 in the Wallingford laboratory, and one in October 1996 in the Teterboro laboratory. In each study, human serum pools were assayed in duplicate once daily for 20 days. The Wallingford study used three pools: one with a low TIBC concentration (~30 μmol/L), one with a normal TIBC concentration (~50 μmol/L), and one with a high TIBC concentration (~70 μmol/L). The Teterboro study used two pools: one with a saturation of TRF <15%, and one with a saturation of TRF >50%. All pools were generated from discarded samples of serum that had been separated from cells by centrifugation of a barrier tube soon after blood had been collected. Once assembled, each pool was aliquoted into multiple 5-mL plastic vials and stored frozen (−30 °C) until used.

BIAS STUDIES

Bias was assessed in two independent experiments (April 1996 and January 1997). In each experiment, three frozen reference pools (9114, 9115, and 9116) were obtained from the CDC. The TIBC and serum iron concentrations of each pool were previously defined by CDC with a reference AutoAnalyzer method *[8]* based on the procedures of Giovanniello et al. *[9]* and of Ramsey *[10]*. We measured the TRF concentration of each pool immunologically on Behring and Beckman equipment at the Teterboro and Wallingford laboratories, respectively. The pools were also assayed colorimetrically on Olympus equipment at the Teterboro and Wallingford laboratories.

PATIENT SAMPLE SELECTION

We studied 570 serum samples from 491 patients. Study samples were selected from serum samples submitted for analysis of iron and TIBC concentrations to each of the three laboratories (Teterboro, Horsham, and Wallingford) in the Quest Diagnostics laboratory network (formerly Corning Clinical Labs.). Samples were specially selected to include a wide range of TIBC and TRF concentrations as well as high and low percent saturation of TRF with iron. Three independent studies of patient samples were conducted at the Teterboro laboratory over 13 months; one study was conducted at the Horsham laboratory over 2 weeks, and one study was conducted at the Wallingford laboratory where 72 samples were assayed for TRF by the Beckman immunologic assay; 52 of the 72 were also assayed for TRF by the Behring immunologic assay. Patient samples from the Horsham and Wallingford laboratories were also analyzed for TIBC and TRF at the Teterboro laboratory.

Table 1 lists the selection criteria used in each of the five studies, and Table 2 lists the range of values for TRF and TIBC, percent saturation of TRF, and the ratios of TIBC to TRF encompassed by the five studies. The mean ratio of TIBC to TRF for 567 patient samples (570 − 3 outliers) was 22.3—not the ideal ratio of 25.0. The TIBC/

Table 1. Selection criteria for 570 patient samples included in five independent studies of paired TRF and TIBC assays and the instruments used for the assays.

Study	Laboratory	Date	Criteria	Instruments
1	Teterboro	Feb '95	Saturation <16% or >49%, or TIBC <45 μmol/L or >70 μmol/L.	TRF Behring, TIBC Hitachi
2	Horsham	Sep '95	Half the samples had saturation values <16% or >49%. The other half had normal % saturation values.	TRF Behring, TIBC Hitachi
3	Teterboro	Sep '95	Same samples used in study 2, but run in a different laboratory on a different Hitachi analyzer two weeks later	TRF Behring, TIBC Hitachi
4	Wallingford	Mar '96	27 samples with serum ferritin <20 μg/L, and 25 with ferritin >250 μg/L.	TRF Behring, TIBC Olympus
5	Teterboro	Mar '96	Wide variation in serum ferritin. 10 samples <50 μg/L, 11 between 50 and 200 μg/L, 10 between 201 and 1000 μg/L, and 10 >1000 μg/L.	TRF Behring, TIBC Olympus

Table 2. Characteristics of the 567 patient samples used in five independent studies.[a]

				TRF, g/L		TRF, % sat.		TIBC, μmol/L		TIBC/TRF ratio	
Study	Laboratory	Date	n	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1	Teterboro	Feb '95	280	2.72	0.71–4.52	20	3–95	60.1	20.0–99.5	22.2	19.3–28.4
2	Horsham	Sep '95	98	2.63	0.75–4.47	34	4–100	57.8	21.3–89.7	21.8	18.7–26.0
3	Teterboro	Sep '95	99	2.63	0.75–4.47	33	4–98	57.9	21.3–89.7	22.1	17.6–28.5
4	Wallingford	Mar '96	52	2.37	0.56–3.82	38	3–100	50.9	12.4–86.5	21.7	19.1–23.8
5	Teterboro	Mar '96	38	2.23	1.02–3.94	33	6–100	52.8	27.6–91.8	24.2	21.5–29.2
	Totals		567	2.63	0.71–4.52	27.2	3–100	57.9	12.4–99.5	22.3	17.6–29.2

[a] Three outliers were excluded from the original set of 570 samples.

TRF ratio of the 567 samples ranged from a low of 17.6 to a high of 29. This wide range can be attributed to the inherent imprecision of Behring's immunoassay for TRF, and to the unavailability of standardized human-based calibrators for UIBC. The TIBC/TRF ratios of the three outliers were 15.2, 13.8, and 10.0; the TIBC concentrations were 43.7, 36.5, and 41.0 μmol/L; and the TRF concentrations were 2.87, 2.64, and 4.09 g/L, respectively.

Results

PRECISION

In two independent studies, the CV of the colorimetric assay for TIBC ranged from 2.1% to 4.1% (two experiments, five pools), the CV of the Behring end-point nephelometric immunoassay for TRF ranged from 4.4% to 6.0% (two experiments, five pools), and the CV of the Beckman kinetic nephelometric immunoassay for TRF ranged from 2.1% to 2.9% (one experiment, three pools).

BIAS

When CDC's target values for TIBC were muliplied by 25.0 we obtained a theoretical TRF concentration that was in close agreement with the immunologically measured TRF concentration as shown in Table 3. For the three pools, the ratio of CDC's target concentration for TIBC to our measured TRF concentration ranged from a low of 23.6 to a high of 24.7. This is close to the theoretically expected ratio of 25.0, and confirms the validity of using the concentration of TRF to check the bias of the TIBC assay. Similar results were obtained in two separate studies that were 10 months apart. Each study was carried out in two laboratories. Table 3 also demonstrates that all laboratories that follow current Hitachi and Olympus recommendations for measuring UIBC are underestimating TIBC by ~8–14% when compared with expected values based on CDC reference pools. This a generic problem with the manufacturers' methods and with currently available calibrators from manufacturers. The individual data points in these bias experiments also reconfirmed the higher imprecision of the Behring immunoassay for TRF when compared with the Beckman immunoassay.

Table 3. Data from two separate studies in each of two laboratories comparing target and measured values (mean of 5) for reference pools from the CDC.

CDC pool	Where measured	Iron, μmol/L	TIBC, μmol/L	TRF-Beckman, g/L	TRF-Behring, g/L
#9114		**Target 13.4**	**Target 45.8**	**Target 1.83[a]**	**Target 1.83[a]**
	Wallingford Apr '96	13.2[b]	41.8	1.94	1.93
	Wallingford Jan '97	12.9	41.2	1.99	
	Teterboro Apr '96	13.2	44.4		
	Teterboro Jan '97	13.3	44.1		2.12/2.00[c]
#9115		**Target 17.7**	**Target 63.5**	**Target 2.54**	**Target 2.54**
	Wallingford Apr '96	17.4	56.2	2.57	2.51
	Wallingford Jan '97	16.8	54.5	2.60	
	Teterboro Apr '96	17.7	57.5		
	Teterboro Jan '97	17.3	57.8		2.81/2.64
#9116		**Target 25.9**	**Target 90.4**	**Target 3.61**	**Target 3.61**
	Wallingford Apr '96	25.1	78.4	3.70	3.78
	Wallingford Jan '97	24.2	77.7	3.67	
	Teterboro Apr '96	24.3	77.5		
	Teterboro Jan '97	25.0	81.7		3.74/3.84

[a] CDC TIBC target divided by 25.0.
[b] Mean of five results.
[c] Mean of five results on each of two days (1st day mean/2nd day mean).

PATIENT CORRELATIONS

Figure 1 is a scatter plot of 567 comparisons of TIBC by colorimetric assay and TRF by Behring nephelometric immunoassay, representing the combined results of five independent experiments in three laboratories. Three outliers were excluded. The correlation between TIBC measured colorimetrically and TRF measured immunologically over a wide range of values is high: $r^2 = 0.941$.

We estimated the contributions of the variances of the TIBC and TRF assays to the total variance of the scatter plot as follows:

The SE of predicted y from $x = S_{y|x} = 3.57$; the mean for $y = 57.8$ μmol/L

$$\therefore CV_{Sy|x} = 3.57/57.8 = 6.18$$

$$CV_y = 3.6; CV_x = 5.1$$

$$\text{Variance of } CV_{Sy|x} = (CV_{Sy|x})^2 = 6.182 = 38.1$$

$$\text{Variance of } CV_y = 3.6^2 = 13.0; \text{ variance of } CV_x = 5.12^2 = 26.0$$

$$\text{Variance of } CV_{Sy|x} = \text{Variance } CV_y + \text{Variance } CV_x + \text{Residual Variance}$$

$$\therefore 38.1 = 13.0 + 26.0 + \text{zero residual}$$

Our estimate for the residual variance is essentially zero. This supports our hypothesis that the observed scatter is secondary to imprecision inherent in the assay and not to any biological variability in the binding of iron to TRF.

Figure 2 is a scatter plot of the results of 72 comparisons of TIBC by colorimetric assay and TRF by Beckman nephelometric immunoassay. The correlation is improved when TRF is assayed with the more precise Beckman assay: $r^2 = 0.968$.

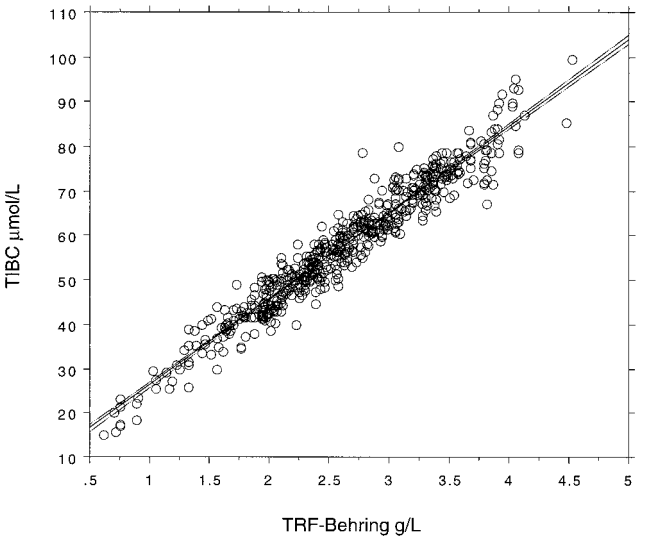

Fig. 1. Scatter plot comparing TRF measured immunologically on the Behring nephelometer and TIBC measured colorimetrically on either Hitachi or Olympus high-volume chemistry analyzers.

n = 567 (three outliers excluded). TIBC μmol/L = 6.90 + 19.41 × TRF g/L. r^2 = 0.941.

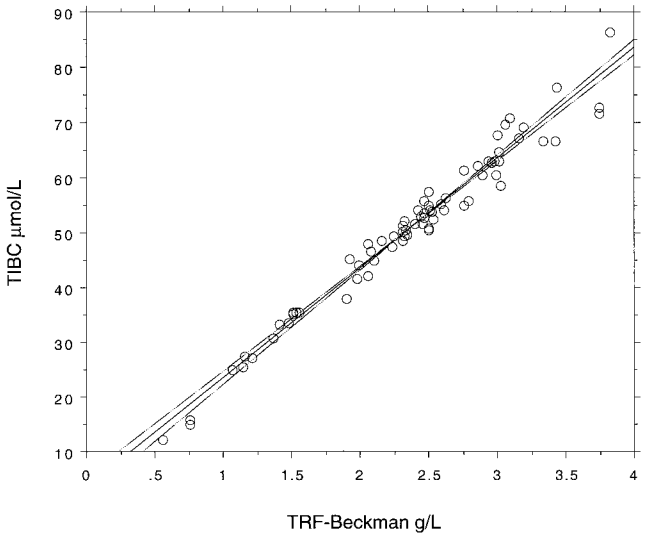

Fig. 2. Scatter plot comparing TRF measured immunologically on the Beckman nephelometer and TIBC measured colorimetrically on the Olympus high-volume chemistry analyzer.

n = 72. TIBC μmol/L = 3.45 + 20.06 × TRF g/L. r^2 = 0.968.

Discussion

Our results support the comparability of TIBC measured colorimetrically and TRF measured immunologically over a wide range of values. We found no evidence of any unusual patient- or disease-related effect on the bias of either the TIBC or the TRF assay. That does not mean that it might not happen, but if it does it is a rare event.

There is wide scatter in literature-reported ratios of TIBC to TRF, ranging from a low of 11.3 *[5]* to a high of 34.4 *[4]*. Only two *[1, 11]* of six other studies *[1, 3, 7, 11–13]* reported ratios close to 25.0 (i.e., 24.4 *[1]* and 23.3 *[11]*), and then only when results in the older study *[1]* were corrected by us to be equivalent to those obtained with the new IFCC TRF standard. Most significant in our analysis of these disparate ratios is the high proportion of observations that differ from ideal when radial immunodiffusion was used as the immunologic method for TRF. Manual radial immunodiffusion methods are not as precise as automated nephelometric methods *[1]*.

Indeed, our results and our review of published articles suggest that any reported deviation from the theoretically expected ratio of 25.0 between TIBC and TRF indicates a problem with either or both assays. Moreover, unless precise and unbiased TIBC and TRF methods are used, identifying a genuine occurrence of "deviant" TRF or TIBC will be impossible.

The results we obtained for the CDC pools with our colorimetric UIBC assay on Hitachi and Olympus equip-

Table 4. Effect of changes in serum ferritin, UIBC, TIBC, and percent saturation on the ratio of TIBC to TRF when chemistry analyzer is calibrated routinely and TRF is measured with a Beckman nephelometer.

Variable	n	Ratio when variable is highest	Ratio when variable is lowest	Δ
Ferritin	52	23.4	21.4	2.0
UIBC	72	20.7	22.4	1.7
TIBC	72	20.8	22.5	1.7
% Saturation	72	22.5	21.1	1.4

ment suggest a manufacturer-related error in the calibration of the UIBC channel, but not of the serum iron channel.

We studied the effect of changes in ferritin, percent saturation of TRF, UIBC, and TIBC on the ratio of TIBC measured colorimetrically to TRF measured on the more precise Beckman nephelometer and found that there is about a 10% difference in the ratio between the lowest and highest values of the variables studied. Table 4 summarizes our findings. The highest ratios were found when ferritin was high, when percent saturation was high, when UIBC was low, and when TIBC was low. There is some selection bias in the ferritin data because only 52 of the 72 samples had a ferritin test, and 27 of the 52 ferritin concentrations were >1000 μg/L. There is a common denominator, however, among all of these variables: the number and availability of binding sites for iron on TRF. When ferritin concentrations are increased, the percent saturation of TRF is usually also high and available binding sites are decreased. When the number of unsaturated binding sites decreases, less time is required for the reaction to approach completion, which results in reporting a higher UIBC concentration, a higher TIBC concentration, and a higher ratio of TIBC to TRF. Conversely, when the number of unsaturated binding sites increases, more time is required for the reaction to approach completion, which is the case when percent saturation and ferritin concentrations are low, and when UIBC and TIBC concentrations are high.

Our results suggest that TRF-containing protein-based calibrators should be used for the primary calibration of Hitachi and Olympus UIBC. A preliminary study of calibration methods at our Teterboro laboratory confirms the superiority of two-point calibration with human serum. Two-point calibration of the UIBC channel with the low and high reference pools from CDC, rather than with an aqueous zero and a single-point commercially available bovine calibrator, yielded superior results. The mean ratio of TIBC to TRF was 24.6 when we assayed 29 different patient-derived minipools covering a wide range of TIBC concentrations. The spread of values for the ratio was very tight, ranging from a low of 23.5 to a high of 26.3, and the correlation of TIBC to TRF was high: r^2 = 0.992. Moreover, two-point calibration of the UIBC channel with human serum eliminated the effect of UIBC concentrations on the ratio.

On the basis of these results, Quest Diagnostics is restandardizing all of its UIBC/TIBC methods to be in agreement with TRF concentrations measured immunologically. In addition, we are working with suppliers of reagents and calibrators to assist them in the development of unbiased secondary nonhuman calibrators for UIBC that are traceable to human-based primary calibrators. The UIBC/TIBC concentrations of the primary calibrators are defined by chemical reference methods and by TRF assays standardized to the IFCC's preparation for plasma proteins.

In summary, TIBC and TRF are equivalent when each is measured precisely and without bias.

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