Evaluation of new immunoenzymometric assay for measuring soluble transferrin receptor to detect iron deficiency in anemic patients

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During the last few years the measurement of serum-soluble transferrin receptor (sTfR) has been introduced as a tool to detect iron deficiency and as an analyte to differentiate between anemia caused by iron deficiency (IDA) and that caused by chronic disease (ACD). Commercially available methods have emerged to make diagnostics by sTfR more readily accessible. We documented the analytical performance of a newly introduced IDEaTM sTfR immunoenzymometric assay (IEMA) by Orion Diagnostica. We also evaluated its clinical performance in 98 consecutive anemic patients, with information derived from bone marrow aspirate samples as the reference for iron status. The clinical usefulness of two other commercially available sTfR assays was assessed for comparison. The analytical performance and clinical applicability of the IDEa were sufficient to support reliable clinical work. We conclude that IDA and iron deficiency in the presence of inflammatory states can be differentiated efficiently from ACD with this new commercial test to measure sTfR.

Iron plays a vital role in the maturation, growth, and division of cells [1-4]. The extracellular transport and dynamic storage of iron within the body is accomplished by its binding to a specific carrier protein, transferrin [5]. Virtually all mammalian cells except the mature erythrocytes have a surface receptor that mediates the flow of transferrin iron from this extracellular pool into body cells by receptor-mediated endocytosis [6-9]. The amount of this transferrin receptor (TfR)3 reflects the potential for cell proliferation. The receptor is composed of two 760-amino-acid glycoprotein subunits of ~95 kDa [7, 8, 10], the functional receptor being a disulfide-linked dimer of two such subunits [11]. The genome for both the receptor and transferrin itself has been located on chromosome 3 [12].

Animal and clinical studies indicate that serum concentrations of the soluble TfR reflect the total mass of tissue receptor [13]. The serum-soluble transferrin receptor (sTfR) is a truncated form of the previously described intact receptor, lacking the cytoplasmic and transmembrane domains [14]. It exhibits a molecular mass of 85 kDa and forms a complex with transferrin in serum. The main sources of the sTfR are the erythropoietic cells of the bone marrow and the circulating reticulocytes that eventually shed their receptors during maturation [15]. Other tissues that contain considerable amounts of TfR, such as the liver [16] and the placenta [17], contribute only slightly to the sTfR pool [7]. Thus, the concentrations of sTfR correlate directly with erythropoietic activity and inversely with the amount of iron available for erythropoiesis, providing a quantitative measure of functional iron status [18, 19].

Confirmation of the absence of iron stores in a bone marrow aspirate sample is still considered the gold standard for the diagnosis of iron deficiency, yet demand for a less strenuous and noninvasive method is evident. In rheumatoid arthritis and other inflammatory diseases, episodes of anemia occur frequently, and the importance of differential diagnostics is pronounced. The quantitation of sTfR has been shown to differentiate effectively between iron-deplete and iron-replete anemic states, irrespective of the presence of acute or chronic inflammatory reactions [18, 20-25]. This finding emphasizes the clinical usefulness of the quantitation of the circulating receptor, as ferritin is a known acute-phase reactant [26, 27], and concentrations of serum iron and transferrin or total iron binding capacity are known to decrease in the presence of an acute-phase reaction [28].

Although the main focus is toward the detection of iron deficiency in anemic patients, other conditions affect the amounts of sTfR as well. Disorders such as autoim-
mune hemolytic anemia, sickle cell anemia, hereditary spherocytosis, β-thalassemia, and polycythemia vera have been reported to increase the number of circulating receptors [29], whereas idiopathic hemochromatosis has been associated with decreased values [30].

Binding assays with radiolabeled transferrin [31, 32] and ELISA-based assays [33-35] have thus far been used to study the sTfR quantitatively. Both polyclonal [34] and monoclonal [31, 33, 35, 36] antibodies have been directed against the receptor.

The objective of this study was to assess the analytical and functional quality of a newly introduced IDEATM TfR immunoenzymometric assay (IEMA) by Orion Diagnostica. Also, the purpose was to show that the concentrations of sTfR measured by this new kit can be used effectively in the differential diagnosis of iron-deficiency anemia (IDA). We also compared this new method with two other commercially available reagents: ClinigenTM (R&D Systems) and TfR enzyme immunoassay (Ramco Laboratories).

**Materials and Methods**

**THE ASSAY**

IEMA is a quantitative test designed for in vitro measurement of the concentration of TfR in human serum. The test is based on a noncompetitive sandwich-type assay technique. It uses mouse monoclonal antibodies in immobilized and enzyme-labeled forms directed against the human TfR. The solid phase, i.e., the microtitration wells, is precoated with specific anti-TfR that binds the soluble receptor in the samples and calibrators. This phase requires 20 µL of calibrators or intact samples to be pipetted directly into each of the microtitration wells with 200 µL of assay buffer and to be subsequently incubated on a shaker for 1 h at room temperature (DELFIA® Plateshake, Wallac). After this the plate is washed (DELFIA Platewash). In the second phase 200 µL of enzyme conjugate solution, in which the same anti-TfR is in the enzyme-labeled form, is added to react with the bound antigen. This is followed by another hour of incubation on a shaker at room temperature, after which the plate is rewarshed. The enzyme used as the label is alkaline phosphatase. In the third phase 200 µL of substrate solution is pipetted to each well, and the enzyme reaction is stopped after 30 min of incubation by the addition of 50 µL of acidic stopping solution into each well. The plate is subsequently incubated on a shaker for 1-2 min, after which the resulting absorbances at 405 nm are analyzed on a SPECTRA® platereader (Wallac).

The amount of bound enzyme-labeled antibody that eventually remains after incubations and washing is directly proportional to the concentration of sTfR in the sample. The actual concentrations in unknown samples are deduced by means of a calibration curve on the basis of 6 calibrator specimens of known concentrations of TfR, which are analyzed in parallel with the samples. The calibrator values span the range of 0.7–11 mg/L. No predilution of the samples is routinely required, yet the instruction manual advises the user to dilute and reanalyze patient samples having initial concentrations above that of the highest calibrator. In these situations the samples can be diluted 1/2 and 1/10 with zero-calibrator serum, a sufficient amount of which is provided in the kit. In this study, however, predilution was performed on patient samples yielding concentrations >7 mg/L, which were discovered to mark the upper limit of the true linear range. The total time required to perform the analysis, including all the necessary preparations and using all of the 96 microtitration wells, is ~5 h. All of the procedures in the course of performing the assay should be carried out within the temperature range of 20–25 °C.

The analyses performed on the Clinigen and TfR enzyme immunoassay methods were carried out according to the instructions provided by the manufacturers and with the same equipment as described above. Patient samples with concentrations above the highest calibrators of the respective assays were diluted and reanalyzed according to the instruction manuals.

**SUBJECTS**

The patient population consisted of 98 consecutive anemic adult patients at the University Hospital of Turku who underwent a bone marrow examination to define the type of anemia and to determine the iron stores. Anemia was defined as a hemoglobin concentration of <128 g/L in men and <17 g/L in women, which constitute the lower 2.5% reference limits in our hospital. All blood samples were obtained before any blood transfusions. Patients on oral iron therapy were excluded from the study population. Patients with hematological malignancies were also excluded from this study, as certain hematological malignancies have been reported to be associated with increased serum TfR concentrations regardless of the iron status of the patients [19, 34]. Additionally, patients who had hemolytic anemia or defined deficiency of vitamin B₁₂ or folic acid were excluded from our study population, because these conditions may be associated with increased sTfR concentrations irrespective of iron status [37]. The patients were assigned to one of three groups on the basis of the bone marrow examination and clinical data. Forty-five patients, who fulfilled the morphologic criteria of iron deficiency and who had no stainable iron in the bone marrow, were classified as having IDA. Thirty-six patients, who presented with stainable iron in the bone marrow, were classified as having anemia of chronic disease (ACD). Of these 36 patients, 23 had recurrent or chronic infections, whereas the remaining 13 had other chronic diseases, i.e., nonhematological malignancies and inflammatory diseases such as rheumatoid arthritis. Another 17 patients, who had no iron in the bone marrow together with an infectious disease, a chronic inflammatory disease (rheumatoid arthritis or ulcerative colitis), or a nonhematological malignancy were placed in a group of combined anemic states (COMBI). These
patient populations are a subfraction of the patient populations in which sTfR concentrations have been evaluated recently with the R&D sTfR assay [25]. The serum samples of 119 apparently healthy nonanemic subjects (23 men and 96 women, ages 24–69 years) were obtained and used as controls.

STATISTICAL ANALYSES
The reference range was calculated from the healthy population by the nonparametric method according to IFCC recommendations with SAS® 6.10 software (Sas Institute). ROC curves were constituted, and the corresponding areas under the curves (AUCROC) were derived with the GraphROC for Windows software package [38, 39]. GraphROC was also used to determine the optimal decision limit of sTfR for iron deficiency. P values were calculated by a two-tailed t-test with a Microsoft® Windows for WorkgroupsTM software package (Microsoft). The correlation scatter plots and the distribution profiles were calculated and visualized with Fig.P® software (Fig.P Software). The protocol of this study was approved by the Joint Committee of Ethics of the University Hospital of Turku and the University of Turku.

Results

ANALYTICAL PERFORMANCE
Detection limit. The detection limit of the method, i.e., the concentration corresponding to the values of counts at 3 SD above the mean for the zero calibrator, was <0.1 mg/L.

Precision. Intraassay CVs (n = 20) for four serum samples (mean concentrations 1.6–5.8 mg/L) were 3.4–6.3%; CVs calculated from duplicate results in 10 subsequent assays were 4.0–6.1%.

Linearity. Serum samples were diluted with zero-calibrator serum. The mean results (Table 1) were 100.2% of expected (range 84.4–122.2%). The assay was linear from 0.3–7 mg/L (not shown). When the concentration of sTfR in the sample exceeds 7 mg/L, the sample can be diluted 1/2 and 1/10 with zero-calibrator serum and reanalyzed.

Recovery. Recovery of purified TfR added to low serum samples was 91.9–102.4% (mean 96.6%). The evaluation was carried out at concentrations within the linear range, close to the upper limit of the reference range. When the expected concentrations exceeded 7 mg/L, i.e., the upper limit of the linear range, the observed recoveries decreased notably.

Drift. Three serum samples and three controls were pipetted at intervals of 0–25 min to simulate the delay in the course of pipetting. No drift was observed.

CLINICAL PERFORMANCE
We tested the IDeA for its capability to detect iron deficiency, i.e., to differentiate between IDA patients and those with ACD. The COMBI group was included so we could study whether the sTfR concentrations differ from the group with a noncomplicated iron deficiency in the presence of an inflammatory reaction. The mean sTfR in the control group was 2.1 mg/L, and the 95% interval was 1.3–3.3 mg/L.

In the iron-deficient group (IDA) the mean sTfR concentration was 10.9 mg/L (range 3.4–29.0 mg/L). The mean value in the ACD group was 2.4 mg/L (range 1.2–4.2 mg/L), similar to the control values. The corresponding values for the COMBI group were 9.8 mg/L and 2.0–34.0 mg/L. Fig. 1 displays the distribution profiles for each group in relation to the upper limit of the reference range.

The capability of the IDeA method to discriminate between these three groups was studied by means of ROC curves. Subsequently, the corresponding AUCROC depicting the diagnostic accuracy were calculated and compared with results obtained with the Ramco and R & D methods (Table 2). All three methods could well establish the difference between the patient groups.

Correlation coefficients for all patient data (r) and for the linear ranges of the respective methods (rLin) were calculated separately (Fig. 2). All patient samples with initial concentrations >7 mg/L on the IDeA method and above the highest calibrators (5 and 40 mg/L, respectively) on the R & D and Ramco methods were diluted and reanalyzed. We encountered evidence that the linear ranges of the R & D and Ramco assays also had upper limits (3 mg/L for the R & D and 16 mg/L for the Ramco) that were substantially lower than the respective highest

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Table 1. The linearity of the assay as determined by measuring the dilution recoveries by diluting four patient samples (A, B, C, D) with zero-calibrator solution, and by calculating the resulting expected/observed percentages (R%).

<table>
<thead>
<tr>
<th>Dilution</th>
<th>A Measured</th>
<th>A R%</th>
<th>B Measured</th>
<th>B R%</th>
<th>C Measured</th>
<th>C R%</th>
<th>D Measured</th>
<th>D R%</th>
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<td>100.2</td>
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<td>3.2</td>
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<td>99.2</td>
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calibrators. These upper limits of the linear ranges were used to calculate the \( r_{\text{Lin}} \) values.

The methods were observed to correlate fairly well within their respective linear ranges, whereas the overall correlation of the Ramco method with the other two assays was notably impaired (Fig. 2).

**Discussion**

During the last few years the concentration of sTfR has been introduced to detect iron deficiency and as an analyte to differentiate anemia caused by iron deficiency and chronic disease. The analytical performance of the IDeA indicates that it can be used reliably in a laboratory environment to determine sTfR concentrations in human serum. The CV of duplicates was <10% even in the lowest samples of 0.4 mg/L. No substantial drift because of a fluctuation in timing in the course of pipetting was encountered, which has previously been identified as a problem in other studies [23]. Because the optimal decision limit of the IDeA method for iron deficiency was
calculated to be 3.6 mg/L, the linear range (0.3–7 mg/L) can be said to cover the area of pronounced importance in clinical decision-making.

The diagnostic accuracy of the IDEa assay was assessed by analyzing three groups of anemic patients, whose iron status had been confirmed from a bone marrow aspirate, and by comparing their sTfR concentrations with those of apparently healthy volunteers. The concentrations of the ACD subjects did not differ from the reference values (P = 0.85); the values of the IDA and COMBI groups were significantly higher (P <0.00001). ROC curves and corresponding AUCROC were used to further assess the clinical applicability of the assay and to compare its differentiating powers with the Ramco and R & D assays. All three assays could well establish the difference between iron-deficient and iron-replete groups, even in the presence of an inflammatory component (COMBI). A slight overlap was observed between the ACD and COMBI groups.

As shown by the scatter plots, the results obtained by the three different methods correlate, as long as the sTfR concentrations remain within the linear ranges of the respective methods (Fig. 2). The difference between the correlation coefficients of the overall patient data (r) and the data restricted to the linear ranges (rLin) indicates that dissociation in correlation occurs as the values increase beyond the linear ranges. The overall correlation between the IDEa and R & D assay remains satisfactory, and the exclusion of values beyond the linear ranges of the respective methods results in only a minor improvement in correlation. The overall results obtained by the Ramco assay, however, correlate poorly with results given by the other two assays. The rLin values of all three comparisons were similar. The instruction manuals supplied with each assay advise the user to dilute and reanalyze any patient samples that yield concentrations above the highest calibrators of the particular methods. Because the analytical performance of the IDEa was studied extensively, we determined the true upper limit of the linear range and used it as the cutoff value, above which all patient samples were diluted and reanalyzed. We discovered, however, that the linear ranges of the other two methods also had upper limits (3 mg/L for R & D and 16 mg/L for Ramco) that were substantially lower than the highest calibrators of the respective methods (data not shown).

In whole, none of the evaluated assays maintained their linearity throughout the range indicated by the calibrators. All three instruction manuals advise the user to dilute and reanalyze samples that initially yield concentrations above the highest calibrators, and in doing so imply that the highest calibrators in fact represent the upper limits of the linear ranges. Our findings contradict these implications. We believe that the difference between the r and rLin values is mainly a result of the false assumption regarding the extent of linearity on the R & D and Ramco assays and could be evened by implementing the upper limits of the linear ranges as cutoff values for reanalysis.

Even though the performance characteristics and clinical usefulness of the three kits are similar, the actual numeric values differ significantly (P <0.00001) and cannot be compared as such. The lack of uniform, international calibrators is the main reason for this. The methods and reagents used in the calibration processes differ, as do the isolation and purification procedures, by which the calibrations have been obtained. Also, the assay conditions for the composition of the diluents and buffers vary. The characteristic affinities and binding sites of the different monoclonal antibodies also introduce a plethora of sources of interference, and the conditions, in which the antibody binds the analyte, have therefore been tuned specifically. The possible inhibitory effect of serum depends on the initial amount of serum pipetted into the wells as well as on the properties of the antibody and poses therefore various effects on the different assays. In the IDEa method intact samples are pipetted directly into the wells, whereas the other two methods require predilution. We are therefore justified in assuming that the matrix effects typically involved in immunochemistry are responsible for some of the inconsistencies in the interpretation of results obtained by the different sTfR assays.

In a recent study the conventional reference range for ferritin was found to be considerably lower than the optimal decision limit for iron deficiency [25]. These values have generally been calculated from a population that has been determined to be nonanemic on the basis of hemoglobin values only. This is, however, an inadequate measure to exclude the effect of latent iron deficiency on the reference values of ferritin, because anemia is known to develop as a late manifestation of iron deficiency. During this study we have encountered the same problem concerning the reference values of sTfR, because healthy volunteers cannot be subjected to bone marrow sampling. Thus, the actual iron status of the population considered “normal” could not be verified. The optimal decision limit of the IDEa method for iron deficiency, however, differs only slightly from the upper limit of the reference range (3.6 and 3.3 mg/L, respectively). Therefore, the capability of sTfR to differentiate between iron-deplete and iron-replete patient groups seems to be considerably less compromised by this matter than is serum ferritin.

The IDEa sTfR assay has proved to be an analytically accurate and clinically promising tool for future studies concerning the differential diagnosis of IDA. In this study we were able to confirm that by measuring the serum sTfR concentrations a good differentiation between the iron-deplete and iron-replete patient groups is possible, irrespective of the concurrent degree of inflammation. All three methods performed similarly.
References


