Transferrin Saturation and Screening of Genetic Hemochromatosis

To the Editor:

Genetic hemochromatosis is the most frequent inherited disease in Caucasian populations. The gene located on the short arm of chromosome 6 was cloned in 1996 [1] and unfortunately named HLA-H. It encodes a protein that is very similar to HLA class I proteins. Two mutations have been described, but the missense mutation (C 282 Y) is observed in >90% of homozygous patients [2]. The fact that the percentage of mutations is variable from one population to another implies the existence of other genes, and makes a genotypic screening impossible.

In 1997 a consensus conference met in Atlanta to define the best procedure to be followed in screening for genetic iron overload. The assay of serum iron and total iron-binding capacity (TIBC) has been recommended as the first step of a phenotypic diagnosis. The ratio between serum iron and TIBC gives the percentage of transferrin saturation with iron.

TIBC is an indirect measurement of the transferrin concentration. The first method described by Ramsay [3] was recently modified by the same author [4]: Excess iron is added to serum to saturate both binding sites of transferrin, and non-bound iron is removed by adsorption on “light” magnesium carbonate. The adsorption capacity of MgCO₃ is well known to vary from one preparation to another [4, 5]. Checking the ability of each brand to adsorb iron is rather cumbersome. There is no reference method for TIBC; nevertheless, a selected method has been published [5]. In 1990 the NCCLS proposed a method for the assay of serum iron and TIBC and will probably finalize it this year. In between-laboratory comparisons, different results were obtained, depending on the analytical procedure [6].

In some pathological disorders, molecules such as ferritin or desferrioxamine bind added iron [7]; TIBC is falsely increased and does not correspond to the binding capacity of transferrin. In iron overload, non-transferrin-bound iron is often present in serum and leads to inaccurate results in transferrin saturation. From a practical point of view the determination of TIBC is tedious, and the step of saturation and removal of excess iron requires technical skillfulness. Few lyophilized control sera are suitable for assessing the quality of TIBC results [8].

The best approach to measure transferrin saturation is to assay the protein itself by an immunological method. Transferrin is a well-known protein that is easily purified. Immuneuropolymetric or immunoturbidimetric methods are widely used in clinical chemistry laboratories. Their precision and accuracy have been greatly improved by the availability of a new international reference preparation (RPPHS-CRM 470) with assigned values for 14 proteins [9, 10]. This reference material was certified by the Bureau Communautaire de Référence (BCR, Bruxelles) and approved by the US Food and Drug Administration. The standardization of commercial calibrators against it contributes to the improvement of quality in control surveys [11]. For this reason, immunochromat assay of serum transferrin should be preferred to the “old” TIBC. The TIBC of transferrin is easily calculated from molecular mass and binding capacity of transferrin [7].

It must be pointed out that the use of CRM 470 triggers significant changes in the standardized results of some proteins, these being lower by 14% for transferrin [9]. Two important consequences must be borne in mind. First of all, new reference ranges should be determined on a world-wide scale. This is the task of the Committee for Plasma Protein Standardization of the IFCC, until available “consensus” reference ranges have been published [12]. Meanwhile, it is probably advisable that reference ranges be determined in each country. Second, new cutoff levels of transferrin saturation should be applied in the laboratory assessment of iron status, either in depletion or in overload.

References

and clinically correlated estimates for normal and abnormal values, and (d) the low cost of the test, which in some US health programs is <$2.00 [1]. In a March 1997 follow-up meeting, participants reviewed preliminary survey data, including those from the Third National Health and Nutrition Examination Survey (NHANES III) [2,3], to determine whether the TS test should be used more widely for screening. The participants also discussed screening by means of gene typing. Vernet and Le Gall, in their letter to the Editor, are correct in pointing out several errors that can occur in the determination of TIBC, just as similar errors can occur, depending upon the analytical method and in the instrumentation used, in the determination of transferrin. For example, using a kinetic nephelometry method, Stoddard et al. [4] found analytical CVs of 4.2% to 6.6% in the analysis of four control materials, 4.2% to 6.8% in multiple analyses of 21 persons, but up to 8.8% in four other, ostensibly healthy, persons. Of concern to the meeting participants were the issues of proficiency testing and standardization. CDC has investigated the capabilities of laboratories to perform these tests, and to date it has found that the analytical CVs for measurements of TIBC and increased concentrations of ferritin are somewhat larger than those for measurements of Fe [5]. Before 1997, there were no proficiency testing programs available in the US that included the determination of TIBC. Starting in 1997, however, the Chemistry Resource Committee of the College of American Pathologists (CAP) agreed to add TIBC on a trial basis. Lyophilized serum may not always be an optimal matrix, but it provides a place at which to start examining potential variability [6]. The preliminary CAP report on specimens C-01 and C-02 for TIBC indicates that most reporting instrument systems performed similarly with these lyophilized materials and that, although the analytical CVs for TIBC were larger than the analytical CVs for Fe in analyses of the same material, this increased variation could most likely be attributed to the additional separation process (ion-exchange resin, magnesium carbonate, etc.) [7]. Also, although NIST currently offers no human serum standard reference materials characterized for Fe and TIBC, SRM 1598 (Inorganic Constituents in Bovine Serum) does have target values for Fe as measured by flame atomic absorption and instrumental-neutron-activation analysis, and it is a frozen, not a lyophilized, material [8].

As regards standardization, the NCCLS-proposed standard document, Determination of Serum Iron and Total Iron-Binding Capacity [9], proposes the uses of ferrozine as a chromogen, trichloroacetic acid precipitation of protein, and basic magnesium carbonate for the removal of excess saturating solution. Results of serum iron and TIBC determinations by the methods recommended by NCCLS and the International Committee for Standardization in Haematology correlated very well with those from the method used to determine Fe and TIBC for NHANES III [10]. This automated continuous-flow method involved protein removal by dialysis, and the addition of basic magnesium carbonate for TIBC. NHANES results provide national normative data for the US population. Six-year total CVs of assays conducted for NHANES III (1988–1994) were 2.4% to 3.6% for Fe and 2.6% to 4.0% for TIBC. In contrast, there are no such available population-based reference ranges for the immunologic measurement that Vernet and Le Gall recommend; developing such ranges would take a long time and could be quite costly.

Although the TS test has emerged as the currently recommended test for hemochromatosis screening, other tests may replace it over time, including the transferrin test proposed by Vernet and Le Gall. The availability of RPPHS-CRM 470 does represent a critical improvement for standardizing the immunoenzymatic analysis of transferrin. Gene typing tests, currently under investigation, may also hold promise in the future. Assays of unbound iron-binding capacity (UIBC) [1] are more easily automated than those for TIBC, and

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Representatives of the CDC respond:

To the Editor:

In 1996, the CDC sponsored a meeting on iron overload at which methods for screening for hemochromatosis were discussed. The percent transferrin saturation (TS) assay, in which TS is calculated from the serum iron (Fe) and the total iron-binding capacity (TIBC), was the method favored at that meeting for several reasons, including: (a) the familiarity of the test to laboratorians and physicians, (b) the fact that Fe and TIBC assays are usually performed on the same instrument, (c) the availability of population-based

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the sum of UIBC and Fe concentrations theoretically yields TIBC. Recent work by Gambino et al. [11] demonstrated that when Fe and UIBC (summed for TIBC) and transferrin were measured precisely and without bias, the ratio of TIBC to transferrin were measured precisely and without bias, the ratio of TIBC (μmol/L) to transferrin (g/L) is close to the theoretically expected value of 25.0.

Further standardization activities for these analytes may well be required, as it was for cholesterol, if acceptable accuracy and precision are to be achieved. Clearly, additional reference materials and expanded proficiency testing programs are highly desirable. We believe, however, that the TS assay, with Fe/ TIBC or Fe/UIBC, is a viable screening test for hemochromatosis and could be widely used as part of routine physical examinations to detect this condition, which is frequently not diagnosed until its serious sequelae such as cirrhosis, arthritis, and cardiomyopathy occur.

References
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Markers of Myocardial Damage

To the Editor:

We read with interest the editorial by Collinson on the use of cardiac troponins T and I (cTnT, cTnI) as specific markers of myocardial damage [1]. We agree with Collinson when he suggests that there were some inconsistencies in the data presented by Bodor et al. [2]. We, too, were concerned by the use of a system optimized for the measurement of serum troponin T to measure this analyte in extracts of muscle tissue, since there are several dilution and matrix problems to address in this setting [3]. Bodor et al. have previously published data measuring cTnI, using the same extraction method [4]. We would suggest that, if the methods were as described in both of these publications, they appear to be fundamentally flawed. The authors omitted a vital step in the purification process, which renders their data highly questionable.

They prepared two separate fractions, one for soluble cytosolic proteins and one for insoluble myofibrillar proteins. The myofibrillar fraction was then solubilized with 8 mol/L urea. The detergent action of urea denatures the proteins of the myofibrillar complex, causing the components of the complex to dissociate. It is well documented that even low concentrations of urea interfere with the Lowry protein assay [5, 6]. The kit manufacturer, Sigma, does not recommend the use of this assay in the presence of urea. However, Bodor et al. used this assay to determine total protein concentration in the presence of 8 mol/L urea. The consequent problem is illustrated in Fig. 1. In addition, 8 mol/L urea could seriously interfere with antibody binding in the immunoassay for cTnT and contribute to a substantial matrix effect. The myofibrillar fraction should have been desalted before any analytical measurements for either total protein, cTnI, or cTnT were performed. Several studies have been published in which urea was used in the protein purification process. However, urea was removed before analysis [7–9].

We have repeated the method of Bodor et al. with the addition of a desalting step. We measured cTnl (Sanofi) and cTnT (Boehringer Mannheim, second-generation assay) in samples from adult human heart and skeletal muscle. The cardiac muscle contained 122 and 59.0

![Absorbance vs. Urea](https://example.com/absorbance.png)

**Fig. 1.** Absorbance at 600 nm observed with the Lowry method for the measurement of total protein (Sigma kit), in the absence of any protein and increasing concentrations of urea.