

# Assessment of Iron Status

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**Objective:** To review the clinical assessment of iron deficiency and excess.

**Conclusions:** Two key iron-related proteins in the human body are ferritin, which is the iron storage protein, and the transferrin receptor, which controls the entry of iron-bearing transferrin to cells. Intact ferritin and truncated transferrin-receptor molecules are present in serum in direct quantitative proportion to their total tissue content. Ferritin and transferrin-receptor production are precisely and reciprocally regulated at a posttranscriptional level. This is achieved by an iron-responsive element-binding protein that interacts with iron-responsive elements in the mRNA of each, but with contrary effects. Increases in serum ferritin reflect increased storage iron and increases in serum transferrin receptor reflect cellular iron deficits. The combined use of these two measurements allows accurate definition of the entire range of body iron status. This is valid even in situations where assessment of iron status has been notoriously problematic, including periods of rapid growth, in pregnancy, in conditions associated with inflammation, and in trained athletes.

**KEY WORDS:** iron; excess; deficiency; ferritin; transferrin; receptor.

## Introduction

In comparison with the other trace minerals, those who study iron are in a fortunate position having, as they do, well-standardized and well-validated assays of iron status. These assays have, in recent years, undergone progressive interpretative refinement insofar as certain measurements allow for relatively precise evaluation of discreet iron compartments. This is an important concept because much erroneous information is deduced if measurements are used to infer compartment sizes that they do not reflect. Although this seems self-evident, much misinformation arises from such inappropriate applications. The measurements of iron status are the focus of the present review and, more particularly, the combined use of the concentrations of serum ferritin and serum transferrin receptor will be highlighted for its ability to portray the entire range of human iron status.

## Body iron compartments

A brief outline is provided of the body iron compartments. This is not intended as a comprehensive overview but, rather, as a framework on which to develop the subsequent concepts. The functional compartment is the iron involved in cellular metabolism. This iron is contained in hemoglobin and myoglobin for the transport and storage of oxygen, is found as components of the electron transport particles that control the step-wise release of energy, is a component of the ribonucleotide reductase enzyme system required for the production of DNA, and functions as a Fenton catalyst in the production of free radicals. The major liabilities of iron deficiency, including impaired psychomotor development, impaired effort tolerance, and adverse pregnancy outcomes are associated only with depletion of this functional compartment (1).

The storage compartment consists of the iron sequestered in relatively safe and nontoxic form as ferritin and hemosiderin. This stored iron represents that absorbed in excess of what is required for the functional compartment. This storage compartment has no known function other than to serve as a repository from which iron, lost from the functional compartment in excess of absorption, is replenished. Recent data have raised the possibility that, if this compartment becomes moderately increased, then its iron may in some way contribute to adverse conditions such as atherosclerosis and neoplasia (2,3). It is well known that the hemochromatotic phenotype is the ultimate endpoint of very marked expansion of this storage compartment.

The storage and functional compartments are linked by a small transport compartment, largely in the form of the carrier molecule transferrin. When the storage compartment has been exhausted and iron is further lost from the functional compartment in excess of absorption, the level of iron in the transport mechanism becomes critically compromised so that progressive functional depletion occurs. When the storage compartment becomes critically overloaded, the transport mechanism becomes fully saturated, resulting in the presence of nontransfer-

rin-bound iron in circulation. This form of iron is readily taken up by parenchymal organs, leading to progressive organ damage and, ultimately, if unchecked, organ failure.

### **Routinely available measurements of iron status**

An array of tests are routinely available with which iron status can be assessed. In terms of the assessment of storage status, however, the serum ferritin concentration is the only readily and widely applicable measurement (4). Although bone-marrow iron, liver non-heme iron content, and quantitative phlebotomy do provide rather precise information, they are too invasive, cumbersome, and impractical for routine application. In the individual case, liver non-heme iron is of value in establishing an unequivocal diagnosis of hemochromatosis.

The percentage of saturation provides an instantaneous reflection of the transport compartment but, by its very nature, it is too labile to be of value as a single determination and, on its own, lacks significant impact in population evaluation.

Although the synthesis of transferrin is controlled by the hepatocyte cellular iron content, the total iron-binding capacity (TIBC) lacks sufficient sensitivity to be used alone as an indicator of functional compartment iron status. Indirect measures of the functional compartment, including free erythrocyte protoporphyrin, mean cell volume, and red cell distribution width, become abnormal relatively late in the development of iron deficiency so that, alone, they are relatively insensitive indicators of the functional compartment (5). In the absence of serum transferrin-receptor measurement, they can be used as an indicator of functional iron status and, together in algorithmic fashion, they can provide useful information for the assessment of population iron nutrition (6). The serum transferrin-receptor concentration provides the single most sensitive indicator of functional iron depletion. It is also the functional depletion marker most likely to become abnormal early in deficiency (5). Finally, hemoglobin concentration, the oxygen-carrying mechanism, becomes reduced as functional compartment depletion becomes well established.

### **Serum ferritin**

Ferritin is produced intracellularly in response to an increase in iron content. This cellular or tissue ferritin is a polymer of heavier, more acidic (H) subunits and lighter, more basic (L) subunits. Depending upon the proportion of H or L subunits in the 24-subunit ferritin polymer, more acidic (H-rich) or more basic (L-rich) isoforms result. These isoforms do have tissue specificity, with the L-rich isoforms tending to predominate in tissues concerned with iron storage. The gene for the H subunit is located on chromosome 11 and that for the L subunit on chromosome 19. The tissue ferritins are not

glycosylated. Cells release a small amount of the ferritin produced into the systemic circulation. By contrast, this serum ferritin is glycosylated and is relatively poor in iron content. The more L-rich serum form is detected by conventional ferritin assays and circulates in plasma in direct proportion to cellular ferritin content. Indeed, over the serum concentration range of 12 to 200 ng/mL, there is a direct quantitative relationship with 1 ng/mL serum ferritin equivalent to 8 mg storage iron (7).

In quantitative phlebotomy evaluation to slowly and progressively deplete the storage compartment, serum ferritin shows a highly predictable decrease in direct proportion to the size of the storage compartment. Once stores are exhausted, the serum ferritin concentration plateaus at a level of approximately 12 ng/mL, showing little further decline as functional compartment depletion progresses (5). As such, serum ferritin is an excellent indicator of the size of the storage compartment, but provides very little information about the functional compartment. Because liabilities of deficiency relate only to functional depletion, serum ferritin provides only an indication of those at risk but no direct information on subjects with functional deficiency. Thus, any study aimed at defining the prevalence of liabilities of iron deficiency should not be based solely upon serum ferritin estimations.

As iron stores increase, serum ferritin concentrations also increase. However, most assays are optimized over the 2–200 ng/mL range. Consequently, use to evaluate significantly higher readings requires either that the assay be reconfigured for the desired range or that additional serum dilutions be performed. In addition, as iron stores rise into the toxic range, parenchymal cell injury results in leakage of tissue ferritin into the circulation. This can be distinguished by additional assay to detect glycosylated ferritin only. This is, however, laborious and not routinely available. These various factors result in ferritin in the very high range being a less-than-accurate indicator of the true size of iron stores.

Additional factors may also confound the interpretation of serum ferritin data. Infection, inflammation, and neoplasia may raise the serum ferritin concentration disproportionately to the iron storage status (4). This relates to ferritin behaving as an acute-phase reactant protein. Alcohol excess and liver parenchymal injury also tend to increase serum ferritin disproportionately to iron stores. Provided, however, that these limitations are appreciated, serum ferritin concentration is the single most applicable, least invasive, and most cost-effective indicator of iron stores. There are certain circumstances where the measurement of serum ferritin, particularly for population evaluation, is only of moderate utility. These relate to situations where stores are low, such as in young children, adolescents during growth spurts, pregnant women, and trained athletes. To maximize information about iron status in these situations, it is imperative to employ an optimal indicator of the functional com-

partment. Reliance on only the storage marker, ferritin, will result in a gross overestimate of functional compartment depletion.

### The serum transferrin receptor

The appreciation that a soluble form of the transferrin receptor is present in human sera in proportion to the magnitude of tissue iron deficit in iron deficiency, has provided us with a uniquely sensitive, early, and highly quantitative evaluation of the functional compartment (8–10). To facilitate understanding of the value of this measurement, the intact receptor and the biochemical identity of the serum form of the receptor will be briefly outlined.

#### THE CELLULAR TRANSFERRIN RECEPTOR: BIOCHEMISTRY

The transferrin receptor has been the subject of a number of excellent reviews (11–13). The transferrin receptor is a transmembrane glycoprotein comprising identical 95 kDa monomers linked by a pair of disulfide bridges. Each monomer consists of 760 amino acids organized into an amino terminal cytoplasmic domain of 61 amino acids, a membrane-spanning segment of 28 amino acids, and a large extracellular domain of 671 amino acids. The cytoplasmic domain is required for appropriate intracellular trafficking and the peptide sequence tyrosine-threonine-arginine-phenylalanine in this region has been identified as the signal for endocytosis *via* coated pits. Serine at position 24 is a phosphorylation site. The transmembrane domain, consisting largely of hydrophobic amino acids, functions as a signal peptide for translocation across the endoplasmic reticulum during synthesis and as a membrane anchor for the protein. Cysteine at position 52 is the major site of posttranslational fatty acid acylation. The significance of this is uncertain but nonacylated receptors undergo more rapid endocytosis. Sites of N-linked glycosylation have been identified at amino acid residues 251, 317, and 727 in the extracellular domain and threonine at amino acid position 104 has been identified as the only site of O-linked glycosylation. These glycans may play a role in translocating the receptor to the cell surface and in facilitating the interaction between the receptor and transferrin. The transferrin binding site is in the extracellular domain, but its specific location and its interacting moieties and those on transferrin are as yet not elucidated. Cysteines at positions 89 and 98 in the extracellular domain are the sites of disulfide linking that hold the homodimers together. The extracellular domain has serine protease-sensitive sites at arginine (100) and arginine (121). Each receptor dimer can bind two transferrin molecules, probably one to each subunit. The gene for the receptor is located on chromosome 3.

#### THE CELLULAR TRANSFERRIN RECEPTOR: FUNCTION

The primary function of the cellular transferrin receptor is to bind to diferric transferrin and to in-

ternalize it by the process of receptor-mediated endocytosis. The affinity of the receptor for its ligand varies at physiological pH in direct proportion to the iron content of the transferrin; being negligible for apotransferrin, moderate for monoferric transferrin, and maximal for diferric transferrin. After binding, the transferrin-receptor complex is internalized in an endocytic vesicle. As this undergoes protonation, iron dissociates from transferrin in the more acidic environment. The liberated iron is transported across the vesicle membrane into the cytoplasm. The apotransferrin molecule remaining has high affinity for the receptor at acidic pH and, by binding, escapes degradation in large measure. The majority of such apotransferrin-receptor complexes are returned to the cell surface *via* rapidly recycling endosomes. At physiological pH pertaining at the cell surface, the affinity is lost and the apotransferrin is released to participate in further internal iron exchange. The transit time through this pathway is rapid, being on the order of 3 to 12 min (11–13).

A minor amount of endocytosed transferrin receptor is processed *via* a different pathway. In this, the endocytic vesicle membrane undergoes multiple internal outpouching resulting in the formation of multiple 50-nm vesicles within a larger vesicle-limiting membrane. The entire structure is referred to as the multivesicular body and the small vesicles as exosomes. These appear to carry transferrin receptors on their surface, with the extracellular domain directed into the cavity of the multivesicular endosome. The multivesicular body is believed to extrude its contents from the cell by the process of exocytosis (14,15). Factors determining which cellular pathway the individual internalized receptor follows remain to be determined.

All body cells express, at some point in development, transferrin receptors on their surface. The highest expression is in tissue, requiring a large and continuous supply of iron. In humans, fully 75% of the total number of transferrin receptors are to be found in or on erythroid precursors in the bone marrow. This number is further increased, either when the functional compartment of iron is reduced or when the erythroid progenitor mass is expanded. The two other major tissues expressing significant numbers of transferrin receptors are the liver and placenta. This latter tissue has been the source of purified transferrin receptor for the production of immunological reagents and for use as a standard in receptor assays.

#### THE SERUM TRANSFERRIN RECEPTOR: BIOCHEMISTRY AND ORIGINS

Soluble transferrin receptor is consistently detected in human serum. Isolated immunoreactive serum transferrin receptors consist of a single protein of 85kDa (cf 95kDa for the intact monomer) (16). It reacts with carboxyterminal-specific antibodies, but not with aminoterminal-specific antibodies (17). By amino acid sequencing, it has been demonstrated to

be a truncated monomeric extracellular domain with the truncation event occurring between arginine (100) and leucine (101). This is consistent with production by proteolytic cleavage. In cell culture models of this phenomenon, it appears that a surface membrane-associated serine protease is responsible for the cleavage (18). The site of maximal proteolysis appears to occur at the surface of the exosome within the multivesicular body prior to exocytosis (19,20). The factors determining which receptors are targeted for such proteolytic cleavage are unclear. Studies showing that diferric transferrin can reduce soluble receptor production indicate a possible effect of intracellular sorting mechanisms as being important controls of production (21). Other data suggest that O-linked glycosylation at threonine (104) may protect the proteolytic cleavage site and that modification of the glycosylation product, as might occur with receptor aging, may open up the site to proteolysis (22). Whether the production of the soluble form of the receptor merely reflects a degradative process or whether it has functional significance is yet to be determined.

#### THE SERUM TRANSFERRIN RECEPTOR: MEASUREMENT

The first reported assay for the measurement of serum transferrin receptor was a two-site immunoradiometric assay (IRMA) using commercially available monoclonal antibodies, OKT9 and B 3-25, developed against the cell surface receptor (23,24). Similar mean values of 251 and 256  $\mu\text{g/L}$  were reported in healthy male and female subjects, respectively. Subsequent assays have used immunological reagents developed against transferrin receptor purified from human placenta. An enzyme-linked immunoassay (EIA) reported by Flowers *et al.* using monoclonal antibodies prepared against isolated transferrin receptor, gave a 20-fold higher normal mean value of 5.6 mg/L with no appreciable difference between male and female subjects (25). This is the assay on which the data for this review are based. The paper in which it is described (25) provides useful information on preparation of reagents and setting up the assay. In studies undertaken to reconcile the discrepancy in values obtained with the IRMA and monoclonal EIA, the difference was shown to be related to whether free or complexed transferrin was used as the standard. The monoclonal EIA gave similar values with the two standards, whereas the monoclonal IRMA varied depending on whether the standard was free or bound. Another assay approach has been to use polyclonal antibodies developed against the transferrin-receptor complex and subsequently absorbed with purified transferrin. Normal mean values with this polyclonal EIA have ranged between 5 and 8.3 mg/L (26).

It is apparent that there are major differences in reported values with different assay systems. Nevertheless, the relative decreases or increases in serum transferrin-receptor values reported in different disease states are remarkably similar. The

20-fold difference in normal values between the monoclonal IRMA and monoclonal EIA is probably related to the fact that the monoclonals in the IRMA were developed against membrane-bound transferrin receptor, which is mainly free rather than transferrin-bound. The antibodies for both the monoclonal and polyclonal EIA were developed against complexed transferrin receptor, which is the form of the transferrin receptor that, presumably, exists in serum. Relatively small differences in reported values with the monoclonal and polyclonal EIA are likely due to minor differences in the purified transferrin-receptor standard, rather than a systematic difference in the specificity of the immunological reagents.

#### Serum transferrin receptor and the assessment of iron status

In all of the serum transferrin receptor assays used, iron deficiency anemia has been shown to be associated with a 3–5-fold increase in concentration with no overlap with normal subjects (23–27). In careful quantitative phlebotomy study (5) to slowly reduce the body iron content, serum transferrin receptor concentrations remain relatively stable within the normal range until the storage compartment has been exhausted. As mentioned previously, this corresponds to a serum ferritin measurement of approximately 12 ng/mL. Beyond this point, as the functional compartment becomes progressively depleted of iron, the serum transferrin-receptor concentration shows a highly consistent and progressive increase in inverse proportion to the magnitude of the iron deficit. Mechanistically, this rise reflects in large measure increased cellular receptor expression per cell but, with severe degrees of iron deficiency, it may be further increased by ineffective erythropoiesis. As functional compartment depletion progresses, the hemoglobin concentration begins to fall, resulting in the development of anemia. In such a careful phlebotomy study, the onset of anemia coincides with a serum receptor concentration of 8.8 mg/L as compared with a baseline average of 5.3 mg/L. Between the point where storage iron depletion occurs and the development of anemia, the only measurement to accurately reflect the iron deficit is the serum transferrin receptor concentration.

As outlined previously, serum ferritin has marked limitations in assessing iron status in young children, adolescents during growth spurts, women during pregnancy, and trained athletes because stores are usually reduced in these groups. Consequently, a marker of the functional compartment should greatly facilitate definition of iron status in these situations. Although further study is required in the evaluation of serum transferrin receptor in children and adolescents, this measurement is of major utility in pregnancy (28) and in trained athletes (Baynes, unpublished observations). This is particularly important in these latter two situations because hemoglobin concentrations are often reduced

due to normal physiological phenomena and are, therefore, of limited help in assessing the functional compartment. Original assay reports suggested that serum transferrin-receptor concentrations were raised in pregnancy (29). This was inferred to occur by solubilization of placental receptors. Subsequent data, however, indicate that serum transferrin receptor is unchanged in pregnancy and an increase only occurs when functional iron depletion occurs (28). This, therefore, allows a much more specific assessment of iron status in pregnancy.

In relation to the other factors that might spuriously elevate serum ferritin concentrations, it appears that serum transferrin-receptor concentration is a more robust measurement. More specifically, acute liver injury and inflammation appear to have no effect on serum transferrin receptor measurements (30). Indeed, serum transferrin receptor measurement is able to distinguish the anemia of inflammation from that of iron deficiency. Prior to the development of this measurement, a bone-marrow evaluation for stainable iron was the only reliable way to make the above distinction. Indeed, combining the data available for anemia of chronic disease (27,30), serum transferrin-receptor concentration gives a positive predictive value for iron deficiency of 91% and a negative predictive value (the proportion of patients with anemia of chronic disease with a normal serum transferrin receptor concentration) of 95%. When combined anemia of chronic disease and iron deficiency anemia coexist, the serum transferrin receptor tracks the magnitude of the functional iron deficit (31,32).

There are, however, certain caveats to the use of the serum transferrin-receptor concentration. Serum transferrin-receptor concentrations may be elevated in the absence of functional iron depletion in conditions of expanded erythroid bone marrow. Such conditions may include hemolytic anemias or ineffective erythropoiesis associated with megaloblastic anemia, myelodysplasia, and hemoglobinopathies such as thalassemia major. In the majority of these, however, serum ferritin concentrations are normal or increased. In addition, hemolytic anemia is associated with reticulocytosis and a normal or increased mean cell volume. Megaloblastic anemia and most myelodysplastic syndromes generally have a raised mean cell volume. Although receptor concentrations may also be raised in situations of effective expanded erythropoiesis, as in primary and secondary polycythemia, this does not present a problem in the assessment of iron status because such subjects are clearly not anemic.

### **The serum transferrin receptor: serum ferritin ratio**

Body iron status can, in the majority of subjects, be precisely defined by two laboratory measurements, the serum ferritin concentration and the serum transferrin receptor concentration. Because the serum receptor remains unaffected during storage

depletion and serum ferritin concentration shows a progressive decline, and because serum receptor shows a progressive increase with functional compartment depletion and serum ferritin remains relatively constant at its reduced level, it would seem to be logical to combine the two measurements in a ratio. Indeed, from quantitative phlebotomy data, the ratio is highly inversely proportional to body iron over the entire range of iron status induced by serial phlebotomy (5). The mean ratio increases from < 100 in the presence of adequate stores to over 2000 at the time of significant functional depletion. The median ratio of 500 is reached at the point of storage depletion.

In preliminary epidemiologic studies of the receptor-ferritin ratio, mean values have ranged from 40 in healthy adult males to approximately 350 in teenage girls, with intermediate values of 151 and 72 in pre- and postmenopausal females (Cook, unpublished observations). The ratio was recently evaluated in an iron-supplementation study in pregnant Jamaican women (33). The median value observed in control women receiving no supplement was 1200, compared to 470 in the supplemented group. The cumulative frequency distribution of the log receptor-ferritin ratio was linear in both control and supplemented women, suggesting that iron status in a population is normally distributed with no separate normal or deficient subpopulations. Clearly, this raises the issue that the use of arbitrary cutoff values for laboratory measurement of iron status to define deficient subpopulations is fundamentally unsound.

The ratio also appears to be of major value in the differentiation of iron-deficiency anemia from the anemia of chronic disease, having an overall diagnostic accuracy of 92%.

### **The biochemical basis for the receptor ferritin ratio**

Because serum ferritin is produced in direct proportion to cellular ferritin synthesis and because serum receptor is produced by proteolytic cleavage in direct proportion to cellular receptor content (34), understanding of the regulation of production of these two proteins may provide insight into how the ratio in serum appears so highly predictable. The cellular content of both proteins is controlled primarily at a posttranscriptional level. This regulation, in the case of ferritin, resides in an approximately 30 nucleotide sequence in the 5' untranslated portion of ferritin mRNA. In the case of the transferrin receptor, this control resides in nucleotide sequences in the 3' untranslated portion of the mRNA. These sequences show significant homology and have been termed iron-responsive elements (IREs). The IREs can be fitted to a consensus motif consisting of a lower stem of variable length, an unpaired cytosine on top of this giving a characteristic bulge, above this an upper stem consisting of 5 complementary base pairs and, finally, a six-base loop is

found atop the upper stem. The first five of these bases are always CAGUG. In the ferritin mRNA, a single such stem-loop structure is to be found at the 5' end and in the transferrin receptor mRNA, as many as 5 such stem loop structures are to be found at the 3' end.

A cytosolic protein has been identified that interacts with the IRE to modulate mRNA translation. This protein is known as the IRE-binding protein or IRE-BP. It is an iron sulphur protein of 90kDa and is encoded by a gene on chromosome 9. In its iron-saturated state (4Fe-4S), it is a cytoplasmic aconitase that lacks IRE-binding activity. When there are less than 4 iron atoms bound, as occurs as cells become iron-depleted, the protein loses aconitase activity but acquires IRE-binding activity. Interaction of IRE-BP with the 5' stem loop of ferritin mRNA prevents the formation of ferritin mRNA polysomes, thereby preventing mRNA translation. Interaction of the IRE-BP with the 3' stem loops of transferrin receptor mRNA in some way masks a rapid turnover determinant in the RNA. The nature of the determinant and of the nucleases are still unclear. The net effect is to stabilize the mRNA, thereby increasing translation. This regulatory mechanism illustrates how cellular iron content through a single cytosolic protein can reciprocally regulate distinct mRNAs (35), thereby leading to a predictable ratio in the serum protein derivatives.

## Conclusion

The serum measurements of ferritin and transferrin receptor are congruent with the observed cellular posttranscriptional regulation of the parent molecules by cellular iron content. Consequently, serum ferritin reflects storage compartment iron and serum transferrin receptor reflects functional compartment iron. Combining these two measurements in a ratio allows an accurate portrayal of body iron over the total range of iron status.

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