Iron Deficiency and Erythropoiesis: New Diagnostic Approaches

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Iron deficiency anemia is one of the most common diseases worldwide. In the majority of cases, the presence of hypochromic microcytic anemia and biochemical evidence for depletion of body iron stores makes the diagnosis relatively straightforward. However, in several clinical conditions, classic biochemical indices such as serum iron, transferrin saturation, and ferritin may not be informative or may not change rapidly enough to reflect transient iron-deficient states (functional iron deficiency), such as the ones that develop during recombinant human erythropoietin (r-HuEPO) therapy. The identification and treatment of iron deficiency in settings such as r-HuEPO therapy, anemia of chronic disease, and iron deficiency of early childhood may be improved by the use of red cell and reticulocyte cellular indices, which reflect in almost real time the development of iron deficiency and the response to iron therapy. In the anemia of chronic disease, measurements of plasma cytokines and iron metabolism regulators such as hepcidin (when available) may be helpful in the characterization of the pathophysiologic basis of this condition. The ratio of serum transferrin receptor (sTfR) to serum ferritin (R/F ratio) has been shown to have excellent performance in estimating body iron stores, but it cannot be used widely because of the lack of standardization for sTfR assays. The combination of hematologic markers such as reticulocyte hemoglobin content, which decreases with iron deficiency, and R/F ratio may allow for a more precise classification of anemias.

Iron deficiency anemia attributable to nutritional deficiency and/or blood loss still remains the most common, treatable anemia in the world. Once the cause for the underlying blood loss or dietary deficiency is identified, the finding of anemia with microcytic hypochromic erythrocytes in conjunction with abnormal serum biochemical indices (low iron, low transferrin saturation, low ferritin) usually leads to the administration of oral iron supplements with improvement of anemia in the vast majority of cases.

In the last decade, major fundamental advances have shed new light on the physiologic and pathologic connections between iron metabolism and erythropoiesis. We now understand in greater detail some of the interactions among the multiple genes and other factors that regulate erythropoiesis (1). The molecular nature and functional properties of the major iron transporters have been identified (2, 3). We have begun to unravel the mechanism responsible for the characteristic microcytic hypochromic features of iron-deficient erythrocytes. Heme-regulated eIF2α kinase (HRI) has emerged as a key regulatory step that controls the synthesis of α- and β-globins in erythroid cells and inhibits the translation initiation factor eIF2α when the intracellular concentration of heme is decreased (4). Additionally, it has been found that suppression of α- and β-globin synthesis by HRI is responsible for the reduction in hemoglobin (Hb) content of iron-deficient erythrocytes. Because they are unable to turn off globin synthesis, iron-deficient HRI−/− mice develop a hyperchromic, normocytic anemia. The entire HRI regulatory mechanism is itself part of a larger complex regulatory system that promotes survival of cells exposed to stressful conditions (5).

New and safer preparations of intravenous iron, such as iron sucrose and ferric gluconate, are now available for the correction of deficiencies unresponsive to oral iron. An expanded repertoire of hematologic and biochemical...
markers is available to assess the balance between erythropoietic activity, erythropoietin (EPO), and available iron.

Major challenges still remain in properly assessing body iron stores and correctly identifying and treating iron deficiency in more complicated settings, such as recombinant human EPO (r-HuEPO) therapy, anemia of chronic disease, and iron deficiency of early childhood. The use of biochemical and hematologic indices in these more challenging situations is the focus of this review.

Assessment of Body Iron
Serum iron, transferrin saturation, and ferritin have been used widely to assess iron status. However, serum iron exhibits diurnal variations, with higher concentrations late in the day, and may transiently reach reference values after the ingestion of meat or oral iron supplements. Serum iron concentrations exhibit variability according to assay methodology and the presence of hemolysis. Oral contraceptives are known to induce increases in serum transferrin and produce inappropriately low transferrin saturation. An approach that uses the ratio of serum transferrin receptor (sTfR) to serum ferritin (R/F ratio) has been shown to have excellent performance in estimating body iron stores (6; 7). A recent study has shown how this ratio can be used to provide accurate and reliable estimates of iron stores in mg/kg of body weight. This was made possible by a careful calibration of the R/F ratio based on quantitative phlebotomy studies (7). Although a single gaussian distribution was observed in US males 20–65 years of age with a mean (SD) body iron content of 9.82 (2.82) mg/kg, two populations were observed in US women: one subpopulation, encompassing 93% of the women, had iron stores of 5.5 (3.35) mg/kg, whereas the other subpopulation had an iron deficit of 3.87 (3.23) mg/kg (7). Changes in R/F ratio were also shown to reflect iron absorption and the true increment in body iron stores after oral supplementation trials (7). In the presence of infection/inflammation, we see inappropriately low concentrations of serum iron and transferrin inappropriately high serum ferritin. Thus, the R/F ratio has limited value in individuals with inflammation or liver disease. It has been suggested that C-reactive protein (CRP) should be used as a screening tool to identify patients with inflammation.

A major laboratory issue that remains unsolved is the lack of proper standardization for the sTfR assay: there are several commercial assays available for sTfR, but no common reference material. Thus, the exciting results of the work of Cook et al. (7) cannot be readily applied to other sTfR methodologies.

r-HuEPO Therapy and Functional Iron Deficiency
The use of r-HuEPO is expanding rapidly, and new preparations with greater potency and longer half-lives are now on the market (8). The initial experience with patients on chronic dialysis (9) and subsequent studies of healthy individuals (10, 11) have shown that, despite the use of oral iron supplements, the increased erythropoietic activity induced by r-HuEPO cannot be sustained by the normally available iron, and iron-deficient erythropoiesis develops. A course of r-HuEPO therapy induces a reduction in serum iron and desaturation of transferrin to values below the threshold of 16% saturation, which is associated with iron-deficient erythropoiesis. This transient discrepancy between marrow iron availability and requirements (defined as functional iron deficiency or iron-restricted erythropoiesis) needs to be promptly identified: changes in r-HuEPO dose or use of intravenous iron can restore an optimal response to r-HuEPO.

In healthy individuals, the amount of iron stores (estimated from serum ferritin) is a powerful determinant of the erythropoietic response to r-HuEPO: baseline serum ferritin values <100 μg/L have been associated with functional iron deficiency in healthy individuals and reduced response to r-HuEPO (11). Unfortunately, serum ferritin concentrations are increased and not indicative of iron stores in hyperthyroidism, inflammation/infection, hepatocellular diseases, malignancies, alcohol consumption, and with oral contraceptives. It is not uncommon to observe response to iron therapy and correction of anemia in patients on chronic dialysis with serum ferritin values in the 200-1000 μg/L range. Thus, additional laboratory markers need to be used to assess the balance between erythropoiesis and available iron. Hematologic indices have been shown to have major value in this setting. The percentage of hypochromic red cells [%HYPO; cells with a mean corpuscular Hb concentration (MCHC) <280 g/L] has been shown to identify the development of iron deficiency in dialysis patients and of functional iron deficiency in healthy individuals treated with r-HuEPO (9, 10). Technical limitations of this marker are related to its sensitivity to temperature and storage (over time, red cells swell, MCHC decreases, and %HYPO increases) and to the inaccurate determination of MCHC by electrical impedance instruments in samples with hypochromic erythrocytes (12). Because of the long life span of red cells, a prolonged state of iron-deficient erythropoiesis is needed to appreciate changes in this marker, which is also increased with simple reticulocytosis. Although previous studies had been negative, (13) %HYPO has been shown to be helpful in predicting the response to r-HuEPO and to intravenous iron therapies (14).

Several reports have been published on the use of reticulocyte counts or other reticulocyte indices for predicting and monitoring response to r-HuEPO. Response to r-HuEPO can be assessed by measuring Hb and reticulocyte counts after 4 weeks of therapy. A change in Hb ≥10 g/L and/or a change in absolute reticulocyte count ≥40 × 10⁶/L identify the patient as a “responder” to r-HuEPO therapy (15). Because 18–36 h elapse between release of reticulocytes into the circulation and their maturation into erythrocytes, studies of reticulocyte cellular indices provide an almost real-time assessment of
the functional state of the bone marrow. Automated analysis of reticulocytes by either hematology analyzers or dedicated reticulocyte analyzers in addition to a much more precise and accurate determination of absolute reticulocyte counts also provides various reticulocyte cellular indices. The additional indices include reticulocyte cell volume (MCVr; in fl), Hb concentration (MCHCr; in g/L), Hb content (CHR; in pg/cell), maturity (based on fluorescence intensity, which is a function of RNA content), and immature reticulocyte fraction (comprising the medium and high fluorescence reticulocytes) (16, 17).

The functional iron deficiency observed in healthy individuals treated with r-HuEPO is associated with early and significant changes in CHR. Healthy individuals with baseline serum ferritin values <100 μg/L produced a sizable fraction of hypochromic, low-CHR reticulocytes when treated with r-HuEPO (11). Use of intravenous iron has been shown to abolish the production of hypochromic reticulocytes and increase the amount of Hb contained in the reticulocyte pool (18).

Several studies have assessed the value of CHR as an indicator of iron-deficient erythropoiesis in patients on chronic dialysis. Earlier studies have been reviewed elsewhere (16). A study comparing iron therapy management with serum transferrin saturation and ferritin to management with CHR showed that there was a substantial reduction in the use of intravenous iron with CHR management (19). A combination of %HYPO >6% and CHR ≥29 pg provided the best discriminative ability to identify responders to intravenous iron therapy (14). Baseline values for CHR and high-fluorescence reticulocytes and their variation after 2 and 4 weeks of intravenous iron therapy allow correct identification of iron deficiency and response to intravenous iron therapy (96% sensitivity and 100% specificity for the latter) (20).

Although promising results have been obtained for the optimization of intravenous iron therapy, the studies published to date on reticulocyte and erythrocyte indices in r-HuEPO-treated dialysis patients have not provided a convincing demonstration that their use can produce a dramatic improvement in the cost-effectiveness of r-HuEPO therapy. Additional studies are needed to clarify this important point, as well as to address the issue of “discordant” biochemical indices in dialysis patients. Many of the patients presenting with serum ferritin values in the high-normal or above-normal range show substantial improvement of anemia with intravenous iron therapy: it is likely that erythrocyte and/or reticulocyte indices could help in managing these challenging cases. The use of CHR to assess iron status in β-thalassemia trait and in α-thalassemias is problematic because these conditions already exhibit an abnormally low CHR. Patients undergoing chemotherapy frequently develop transient megaloblastic/macrocytic erythropoiesis, with production of large reticulocytes with increased CHR. These reticulocyte changes will prevent the identification of functional iron deficiency based on reduced CHR. In addition, the availability of CHR measurements on only one brand of the currently available hematology analyzers severely limits their availability and use.

Although reticulocyte fluorescence maturity indices have shown some promise in identifying engraftment after hematopoietic cell transplantation, the number of well-conducted studies is insufficient to assess their potential value in r-HuEPO therapy.

**Diagnosis of Iron Deficiency in the Presence of Inflammation and the Anemia of Chronic Disease**

The diagnosis of iron deficiency or functional iron deficiency is particularly challenging in patients with acute or chronic inflammatory conditions because most of the biochemical markers for iron metabolism are affected by acute-phase reaction. Recently, a novel approach based on hematologic indices has been shown to provide important new insights into the proper identification of functional iron deficiency and the diagnosis of iron deficiency in the presence of an acute-phase response (21). Using hematologic indices to define iron-deficient states (%HYPO and CHR), the authors were able to assess the value of ferritin and sTfR in patients without and with acute-phase response (based on a CRP cutoff of 5 mg/L). Functional iron deficiency was defined as a CHR <28 pg and a %HYPO >5%, based on the distribution of these values in healthy controls. The biochemical markers performed significantly better in the absence of inflammation: the cutoff for sTfR/ferritin index was 1.5 for simple iron deficiency and 0.8 for iron deficiency combined with inflammation. The relationship between CHR and the sTfR/F index can be described in a simple plot: four quadrants can be identified based on the respective cutoff values for these markers in simple iron deficiency and iron deficiency associated with inflammatory conditions, as shown in Fig. 1 (21). The four quadrants identified in these plots correspond to four different states: (a) iron repletion, normal erythropoiesis; (b) patients with reduced iron supply but not yet in an iron-deficient erythropoietic state; (c) depletion of storage and functional iron compounds, with decreased hemoglobinization of erythrocytes; and (d) functional iron deficiency in iron-repleted state, with decreased hemoglobinization of erythrocytes (21). On the basis of the placement of individual patients in this plot, decisions can be made on the suitability of either iron replacement or r-HuEPO therapy.

The anemia of chronic disease comprises various clinical conditions with prominent signs of chronic inflammation and increased plasma concentrations of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and interferon-γ. Several pathogenetic mechanisms are probably involved in this group of heterogeneous diseases. An important mechanism is based on an increase in erythropoiensis induced by inflammatory cytokines such as TNF-α (22). This leads to anemia attributable to a decreased number of active erythroid progenitors, which can be reversed by
monoclonal antibodies directed against TNF-α (22) or by r-HuEPO. An inappropriately low production of EPO and decreased EPO responsiveness may also contribute to increased erythropoiesis in chronic diseases.

Another important pathogenetic mechanism is based on altered iron metabolism. Decreased values of serum iron, transferrin, and transferrin saturation are seen in conjunction with normal/increased serum ferritin. In addition, iron absorption is reduced, and iron tends to be sequestered into the reticuloendothelial system. In systemic-onset juvenile chronic arthritis, high circulating concentrations of IL-6 have been shown to be associated with defective iron supply to the erythron and microcytic anemia that responds to intravenous iron (23). Proinflammatory cytokines increase the cellular uptake of non-transferrin-bound iron via divalent metal transporter-1, but reduce the iron export by down-regulating ferroportin.

A recently identified 25-amino acid antimicrobial peptide, hepcidin, has been shown to play a major role in iron homeostasis (25). Mice lacking this peptide show progressive iron accumulation (26), whereas overexpression of hepcidin in mice leads to decreased iron stores and severe microcytic hypochromic anemia at birth (27). Hepcidin mutation has been identified in families with severe juvenile hemochromatosis (28). Anemia and hypoxia decrease liver hepcidin expression, providing a possible mechanism for the associated increase in iron absorption and in iron release from stores (29). Acute inflammation dramatically increases hepcidin expression and reduces serum iron twofold in mice (29). Up-regulation of hepatic hepcidin expression correlates with serum ferritin when transferrin saturation is within reference values (30). In vitro data suggest that non-transferrin-bound iron, which is abnormally increased in thalassemias and other hemolytic anemias, hereditary hemochromatosis, and hypothyroidism, may down-regulate hepcidin expression, providing a possible explanation for the increased intestinal iron absorption and the iron overload seen in these pathologic conditions (30). The iron-refractory anemia of patients with large hepatic adenomas is associated with markedly increased hepcidin mRNA concentrations (31). The notion that hepcidin may be a cytokine-regulated gene that impairs both iron recycling in bone marrow/macrophages and intestinal iron absorption has been recently confirmed by the demonstration that its production, as all type II acute-phase reactants, is induced by IL-6 but not IL-1 or TNF-α (32). Studies on hepcidin have been hampered by the inability to measure protein concentrations in plasma. Urinary excretion of this peptide has been assessed recently by immunoblotting using rabbit anti-human hepcidin antibodies (32). Development of a reliable assay for hepcidin could greatly improve our understanding of its role in a variety of pathologic conditions.

TfR has been shown to be of diagnostic value in iron deficiency anemia and in the anemia of chronic disease. This marker is crucial in assessing the relationship between Hb and serum EPO concentrations because it is inversely related to serum EPO concentrations (33). A sTfR value inappropriately low for the degree of anemia is an indication of decreased bone marrow erythropoietic activity (34).

Clinical studies on the anemia of chronic disease and clinical management of these patients would benefit from a better understanding of the underlying cytokine activation patterns. Assays for CRP, EPO, and sTfR are currently available for use on automated chemistry analyzers or random access immunoanalyzers. Most of the assays that could be of value in identifying a particular pattern of inflammatory response in patients with anemia of chronic disease are available only as ELISAs (such as IL-1, IL-6, TNF-α, interferon-γ, and others), which are not suited for general use in clinical laboratories. The clinical applicability of these inflammation markers will be limited unless they can be transferred to automated chemistry/immunoanalyzers suitable for a clinical laboratory.

Fig. 1. Diagnostic plots for the identification of iron deficiency in anemic patients with (left) and without (right) acute-phase reaction.

Data for 288 patients with acute-phase reaction and 154 patients without acute-phase reaction are presented. Data from the Dade Behring sTfR assay are shown. The sTfR-F indices separating patients in the iron-repleted state from those in the iron-depleted state are 0.8 in patients with acute-phase reaction (left) and 1.5 in those without (right). ○, %HYPO < 5; ●, %HYPO > 5; □, inverted CHR/CH ratio; ●, combination of %HYPO > 5 and inverted CHR/CH ratio. Reprinted with permission from Thomas and Thomas (Clin Chem 2002;48:1066–76).
Iron Deficiency in Early Childhood

In the US, 700 000 toddlers 1–2 years of age are biochemically iron deficient; 240 000 of these toddlers have iron deficiency anemia (35). Although anemia can be cured by iron supplementation, altered cognitive performance (lower test scores of mental and motor development) may not be correctable (36, 37). Prompt recognition of iron deficiency, before the development of anemia, may prevent permanent impairment in intellectual development. Infants most at risk for development of iron deficiency are those older than 6 months who are exclusively breast-fed and infants between 9 and 18 months of age if fed with cow’s milk or low-iron-content formulas. After 18 months of age, the risk of iron deficiency is reduced by the exposure to a regular diet and by the reduced rate of growth, which translates into lower iron requirements. Thus, testing for iron deficiency around 9 months of age and around 3 months for premature babies has been advocated to prevent the consequences of iron deprivation (38).

In a group of 210 pediatric patients [mean (SD) age, 2.9 (2.0) years] undergoing routine office visits, CHr emerged as the strongest predictor of iron deficiency and iron deficiency anemia (39). Plasma ferritin was not a predictor of either condition. TfR and erythrocyte zinc protoporphyrin had little or no diagnostic value in this group of young children. Children with CHr <26 pg (optimal cutoff value based on sensitivity/specificity analysis; value for H*3 instruments from Bayer Diagnostics) had lower Hb, MCV, MCH, serum iron, transferrin saturation, and increased red cell width distribution (all with P <0.001). The estimated probability for iron deficiency was >90% with CHr <20 pg. When CHr was close to 23–24 pg, the probability of iron deficiency was ~50%, and this probability decreased smoothly for larger values of CHr. Children with CHr >29 pg had virtually a zero probability of iron deficiency. This study raised the possibility that diagnosis of iron deficiency in early childhood could be achieved exclusively on the basis of hematologic indices, without the need for biochemical studies.

Recent longitudinal studies in infants and toddlers have demonstrated that CHr identifies iron deficiency in infants and toddlers significantly better than does Hb (40). However, optimal CHr values for predicting iron deficiency in infants and toddlers are lower than the adult range (29–32 pg; ADVIA 120 instruments; Bayer Diagnostics). This difference is mostly attributable to the transient physiologic decrease in MCV and MCH observed during the first 2 years of life. A CHr <27.2 pg was a significant predictor of iron deficiency in infants, whereas for toddlers the cutoff value was <28.4 pg (40). Diagnosis of iron deficiency is improved by a careful dietary history: drinking cow’s milk before age 12 months and more breastmilk than formula after 6 months were significantly associated with iron deficiency. A focused diet history combined with Hb or CHr was shown to identify more infants with iron deficiency than either Hb or CHr alone (41).

With the cloning of the major mammalian iron transporters, it is likely that in the near future human mutations will be identified for these transporters (42). In addition, the ongoing studies on gene modifiers of iron metabolism are likely to provide several additional genes that may affect disease phenotype and response to therapy. This has already been shown for women carrying the compound HFE genotype C282Y/H63D: they are less likely to develop iron deficiency because of their increased iron stores and Hb concentrations compared with healthy controls or women carrying only the C282Y mutation (43). Although the diagnosis and treatment for uncomplicated iron deficiency remain straightforward, the increasing number of physiologic or pathologic states associated with abnormal erythropoietic iron metabolism can now rely on a much expanded and refined repertoire of biochemical and hematologic laboratory tests. The challenge for the clinical laboratory is to provide robust and informative assays that can be easily used by clinicians in diagnosing and treating patients with these more challenging alterations in iron metabolism.

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