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Review Article

Diagnostic Approaches to Iron Deficient States and Iron Deficiency Anemia

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ABSTRACT:

Iron deficiency anemia is the most common hematological disorder worldwide and a considerable source of morbidity: it impairs cognitive development in children and substantially affects quality of life in adults. Diagnostic approaches for biochemical iron deficiency in the absence of anemia and iron deficiency anemia share a reliance on both biochemical and haematological markers. While the diagnosis of iron deficiency anemia is straightforward in uncomplicated cases, it is more challenging in the presence of other diseases, or of chronic inflammation/infection, or when erythropoietic stimulating agents are used. In these cases, novel parameters like reticulocyte haemoglobin content and serum hepcidin are useful both for diagnosis and for prediction of response to iron replacement therapy. This review examines a variety of hematological and biochemical biomarkers currently in use or considered for use in the diagnosis and treatment of iron deficient states.

KEY WORDS: *Iron deficiency, Anemia, Diagnosis, Hematological, Biochemical biomarkers*

Assessment of iron status is based on a combination of hematologic and biochemical markers, geared toward identifying iron deficient erythropoiesis and/or unusually low iron stores. Iron deficiency progresses through 3 states, which have their origin in a discrepancy between iron requirements for erythropoiesis and iron availability from body stores and diet/gastrointestinal absorption.*[1] The 3 stages are:

Storage iron depletion: the iron stores are depleted below the normal range but there are no significant hematological changes. Biochemical markers are useful at this early stage.[2,3]

Iron deficiency with no anemia: biochemical and other hematological markers indicate that iron availability to the erythroid marrow is limited, but Hb still remains within the normal range or begins to decrease slightly form baseline steady state values.[4]

Iron deficiency anemia: anemia is present, in conjunction with classical biochemical and hematological signs of iron deficiency.[5]

Diagnosis of the third stage, iron deficiency anemia, is straightforward in most cases, based on the patient's clinical history (poor iron intake and/or blood loss) and the presence of hematologic hallmarks of iron deficient erythropoiesis (reduced Hb, MCV and MCH, inappropriate reticulocyte response for the degree of anemia and low reticulocyte Hb content) in conjunction with biochemical

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*This simplified model, useful as it is, applies only to uncomplicated iron deficient states due to either dietary insufficiency or excessive iron loss; it is not applicable to conditions in which the movement of iron across cellular compartments is altered, as in the case of chronic inflammation or the anemia of chronic disease.

signs of iron stores depletion (low serum ferritin, serum iron and transferrin saturation).

Prompt identification of the earlier stages of this condition can be challenging, especially when other conditions may alter the signals provided by the "traditional" diagnostic criteria, or in infants or toddlers, where progression to iron deficiency anemia may be missed if proper tests are not used. When iron may be present in the body stores, but is not made available for erythropoiesis, diagnosis is especially difficult: most frequently this is due to the presence of a chronic inflammatory state or other pathologies, often combined into the single category of anemia of chronic disease (ACD). In addition, iron supply that meets the baseline requirements of the erythron may not meet the demands of enhanced erythropoiesis. This condition has been defined as iron restricted erythropoiesis or functional iron deficiency.[6] Functional iron deficiency has emerged as an important factor limiting the hematological response to recombinant human erythropoietin (r-HuEPO) or other ESAs.[4,7]

In rare, challenging cases, diagnosis depends on demonstrating responsiveness to IV iron.In the majority of uncomplicated cases, changes in laboratory parameters can be easily identified in individual patients over time. Markers with high biological/analytical variability are obviously less reliable in this setting, and diagnosis must be based on markers with lower variability.Next follows a discussion of individual biochemical and hematologic markers and how they can be properly integrated for optimal disease identification and management.

A. CHEMISTRY BIOMARKERS

A.1. Plasma/Serum ferritin

Intracellular ferritin is a molecular complex formed by the interaction of twenty four H (heavy/heart, 21 kd) and L (light/liver, 19.7 kd) subunits assembled in a spherical form, with thousands of iron atoms stored in its central core. Ferritin in serum is either secreted from iron storage sites or is liberated as a consequence of cell death/lysis; it consists almost exclusively of the L-chain subunits, which are also glycosylated. Serum or plasma ferritin values are measured by immunoassays, with a normal range varying between 20 and 300 ng/mL (or μg/L). The assay is insensitive to mild hemolysis; however, complete hemolysis of the sample may result in a marked increase in serum ferritin due to the release of ferritin contained in the erythrocyte. Serum ferritin has no circadian variation and it is quite stable in the same subject over time (low intra-individual variability).Ferritin values, on the other hand, change with age: they are higher at birth, decrease during childhood and increase with age in adults. It is of interest that from screening in large populations it was shown that in the absence of known mutations associated with hemochromatosis, serum ferritin appear to be higher in Asians and Pacific Islanders both males and females.[8] Several patients have been described with hyperferritinemia in the absence of iron overload, most likely due to mutations not involving the ferritin gene.[9]

Serum ferritin is a reliable and useful marker of iron stores, in the absence of acute or chronic inflammation (serum C-reactive protein < 0.5mg/dL). A value below 12 mg/L is typically diagnostic of complete depletion of iron stores. The sensitivity of such a threshold has been questioned, however, and there is evidence supporting the notion that a threshold of 30 ng/mL would yield better sensitivity with unchanged specificity (positive predictive value of 92% versus 75% for the lower threshold).[10]

In rare conditions, hypothyroidism and vitamin C deficiency may lower serum ferritin levels independently of iron depletion.On the other hand, hyperthyroidism, liver disease (especially due to hepatitis C virus), alcohol consumption and oral contraceptives increase serum ferritin values independently of iron status.[11] In the case of damage of ferritin-rich tissues by acute or chronic processes, serum ferritin increases as a direct reflection of cellular damage. This can be seen in liver disease, leukemias, pancreatic and bronchial cancer, and neuroblastomas. While serum ferritin is normally glycosylated, ferritin released from damaged tissues are not. Serum ferritin is also an acute-phase reactant, which becomes elevated in the presence of acute or chronic inflammatory states. Furthermore in the hyperferritinemia-cataract syndrome, mutations in the iron responsive element of the L-ferritin gene result in elevated serum ferritin values in the absence of iron overload.[12]

In children, serum ferritin is not helpful in identifying iron deficient states and/or the development of iron deficient erythropoiesis.[13,14] In the anemia of chronic kidney disease (CKD), serum ferritin is also not a reliable marker of iron stores, since it is elevated due to the concomitant chronic inflammatory state. Ferritin values > 200 μg/L are usually recommended for dialysis patients,[5] and values of 100 μg/L are considered the lower limit of normal for CKD patients.[16] The sensitivity for ruling out iron deficiency was determined to be 90% for a ferritin cut-off of 300 μg/L and 100% for a 500 μg/L cut-off in CKD patients.[17]

A.2. Serum iron, transferrin, and transferrin saturation

Iron circulates in plasma as transferrin-bound iron, which allows the transport of iron to cell types endowed with specific cell-surface receptors. Most of the assays used

to measure serum iron are spectrophotometric methods based on the complexing of the iron with a chromogenic substrate. Iron is measured after its release from transferrin by acid pH treatment. These methods require the use of either serum or heparinized plasma; samples collected in EDTA are unsuitable for serum iron determination. Less commonly, iron can be directly measured with atomic absorption.It's important to note that serum iron values reveal diurnal variations and respond to dietary intake, resulting in higher serum iron values early in the day or following the ingestion of iron-rich food or dietary supplements. Measurements are best performed in the morning, after > 8 hrs. fasting, to avoid these transient increases in serum iron due to food or multivitamin supplements containing iron.Other situations can influence the measurements as well: serum iron decreases with infection and inflammation;[5] hemolysis may affect some of the biochemical methods used to measure iron and show falsely elevated values;[18] some serum iron assays perform poorly in dialysis patients.[19] A potential reason for the poor performance of some assays may be the release of iron from ferritin, which significantly affects serum iron measurements when ferritin is above 1200 ng/mL.

As its name denotes, transferrin is the carrier for almost all iron circulating in plasma. As an 80-kd glycoprotein, it's capable of binding iron in two homologous domains in the N- and C- terminals of the molecule, and is produced and secreted mostly by the liver. Under normal conditions, transferrin is only 30% saturated by iron.

.Total iron binding capacity (TIBC) is no longer directly measured, but is estimated on a molar basis as being two-fold the concentration of transferrin, which is directly measured with immunoassays.

Transferrin synthesis and values in plasma are increased in cases of iron deficiency, and augmented iron needs, such as those of pregnancy Serum transferrin can be found elevated with the use of oral contraceptives and decreased with inflammation/infection. It is also increased in the presence of parenchymal liver damage.

Several genetic polymorphisms have been described in the transferrin gene, with Caucasians carrying almost uniformly the C allele, and West Africans carrying most frequently the D allele. A study in Zimbabwe, for example,showed that 90% of subjects had the TF CC genotype, and 10% had the TF CD genotype.[20] In normal males with TF CD heterozygotes, significantly lower values for serum iron, TIBC, and TF saturation were observed, suggesting that these individuals may be somehow protected against iron overload.[20]

Due to an anti-transferrin immunoglobulin, elevated serum iron, transferrin, and monoclonal gammopathy of undetermined significance are seen in transferrin-immune complex disease, a rare and acquired disease.[21]

Bainton and Finch showed that a transferrin saturation below 16% is insufficient to meet the functional requirements of the erythroid marrow.[24] The conditions that may alter either serum iron or transferrin values to this degree have been listed above: de-coupled values of iron and transferrin from iron metabolism substantially limit the value of these parameters in assessing the adequacy of iron supply to the marrow.

A low baseline serum iron has been shown to be an independent predictor of increased mortality and hospitalization in dialysis patients,[25] whereas increased TSAT was associated with lower mortality.[26] Fishbane *et al* have examined 34,782 NHANES III patients for whom TSAT and ferritin data were available:[27] more than 50% of the patients had values below either the ferritin (100 ng/mL) or TSAT (20%) thresholds. Additionally, they found that,the prevalence of iron deficiency increased with deteriorating renal function in men but not in women.

A.3. Serum values of soluble Transferrin Receptor (sTfR)

The soluble transferring receptor (sTfR)is particularly useful in distinguishing the anemia of chronic disease, which has normal or near normal sTfR values from iron deficiency anemia (elevated sTfR).[10,28-31]

sTfR is a truncated form of TfR, consisting of the extracellular transferrin-binding domain of the molecule, which is cleaved from the rest of the molecule by a specific protease between aa 100 and 101. sTfR loss from the maturing erythroid cells is an indirect expression of the activity of the erythron and is not directly affected by inflammation. Independently of iron status, sTfR will increase in hyperproliferative anemias, with the use of ESAs, and decrease in hypoproliferative states, due to either EPO deficiency (chronic renal failure) or marrow aplasia.[31] Independently of erythroid marrow activity, sTfR values are abnormally elevated in iron deficient states, reflecting a upregulatory response driven by the limited availability of iron to the erythron;[32] conversely, they are depressed in the presence of iron overload.[33]

A variety of methods have been employed to measure sTfR, all based on immunoassays and with one method suitable for both whole blood and serum/plasma assays.[34] Unfortunately, there is not yet a single reference standard, and different methods generate values with different units and normal ranges, as highlighted in a recent commutability study.[35] A whole blood assay is available for sTfR, but its utilization has been limited.[34]

The effort to measure sTfR values seems promising for some cases. sTfR values <6 mg/L (NV 3.8-8.5) were shown to be predictive of response to EPO therapy in anemic dialysis patients.[36] Because increased erythropoiesis

increases sTfR, however, this parameter does not detect functional iron deficiency. Other studies have failed to show a predictive value of sTfR in CKD anemia management.[37,38] A decline in sTfR may reflect increases in iron availability, as seen when intravenous ascorbic acid is used to mobilize iron stores in dialysis patients.[39]

Transferrin receptor type 2 (TfR2) is a homologue of the classic type-1 TfR (discussed in section A.3) which exhibits lower affinity for transferrin, and together with TfR1 plays a crucial role in iron metabolism.[22] Mutations in the TfR2 gene are found in recessive hemochromatosis type 3.[23]

A.4. sTfR-Ferritin (sTfR-F) index

Efforts to measure sTfR were advanced by Skikne *et al.*, whose research demonstrated that the log ratio of sTfR to ferritin correlates quite well with iron stores.[32] This approach has yielded precise estimates of body iron in large epidemiological studies.[1] The log ratio of sTfR to ferritin identified more children and fewer females as iron deficient compared with ferritin alone.[40] A recent study has shown that sTfR/ferritin and ferritin are equally satisfactory in quantifying iron stores in population studies, with ferritin having a lower cost and a better availability of reference material.[41,42] There is limited evidence to support the use of this or similar indexes, however, in dialysis patients,[43,44] a slightly different ratio (sTfR/log ferritin) has also been used to identify iron deficient states,[30,45,46] For the sTfR to log ferritin ratio, cut-off values of 1.8 (90% C.I. 1.55 to 1.99) and 2.2 (90% C.I. 1.81 to 2.53) have been proposed to identify iron storage depletion and iron deficient erythropoiesis, respectively.[45] ICut-off values of 1.5 and 0.8 have been proposed for the sTfR/log ferritin for the diagnosis of iron depletion in the absence and presence of inflammation, respectively.[46] However, since these cut-offs are sTfR assay-specific they can be as high as 3.8 and 2.0, respectively, when a different assay is used.

A.5. Urinary and serum Hepcidin values

The identification of hepcidin as a key regulator of iron homeostasis has added a new biomarker for the diagnosis of both iron overload and iron deficient states and thus serum hepcidin determination may help shed light in patients' iron status.[47] Hepcidin is a 25 aa peptide produced and secreted by the liver, which modulates iron availability by causing internalization and degradation of ferroportin, a key iron exporter, which is essential for both iron absorption in the duodenum and recycling of iron/iron efflux by macrophages. Hepcidin is a negative regulator of iron absorption and mobilization: high hepcidin values turn off both duodenal iron absorption and release of iron from macrophages; low hepcidin values promote iron absorption and heme iron recycling/iron mobilization from macrophages. Thus, hepcidin values are expected to be high in iron overload states and diminished in iron deficient states (Table 1). In normal subjects, an oral iron loading produces a measurable increase in hepcidin values.[3] while EPO therapy produces a reduction in serum hepcidin values, which may be indicative of EPO-responsiveness. Early studies focused on quantifying hepcidin in the urine or serum with mass spectrometry detection.

An immunoassay for serum human hepcidin (lower limit of detection: 5ng/ml) produced normal range values of 29-254 ng/ml in men and 16-288 ng/ml in women.[3]

TABLE 1. Hepcidin, diagnostic applications in anemias

Hepcidin	Condition	Biochemical markers	Hematological markers
Low	Iron deficiency	↓ iron,Ferritin TSAT ↑ sTfR	↓ Hb, MCV, MCH, CHr ↑ RDW
Low	Ineffective erythropoiesis/iron loading anemia	↑ iron, Ferritin, TSAT ↑ sTfR	↓ Hb, ↑ RDW
High	Anemia of chronic disease (ACD)	↓ iron,TSAT ↑ ferritin	↓ Hb, MCV, MCH, CHr ↑ RDW
High	Anemia of chronic kidney disease	↓ iron,TSAT ↑ ferritin	↓ Hb, MCV, MCH, CHr ↑ RDW
Norm-High	Anemias on chronic transfusion	↑ iron, Ferritin TSAT ↓ sTfR	Variable

A new sandwich ELISA assay has brought the limit of quantification for hepcidin down to 10 pg/mL.[48] The assay sensitivity allows for the detection of changes in serum hepcidin due to diurnal variation and in response to oral iron. Measurements of prohepcidin, the precursors of the biologically active 25 aa hepcidin, are poorly correlated with hepcidin and are unresponsive to known hepcidin regulators.[49] In iron refractory iron deficiency anemia (IRIDA), an increased serum Hepcidin is accompanied by low serum ferritin values, a unique combination of high diagnostic significance.[50,51] Studies have found that elevated serum hepcidin values may predict poor therapeutic response to oral iron[51,52] and to ESA therapy.[53,54]

A.6. Erythroferrone (ERFE), Growth differentiation factor 15 (GDF15) and Transmembrane serine protease 6 (TMPRSS6)

The erythroid regulator erythroferrone (ERFE) has been shown to be a key mediator of the EPO effects on hepcidin production.[55] With increased erythropoietic activity, ERFE down-regulates hepcidin production, potentially explaining the iron-overload characteristically associated with ineffective erythropoiesis.[56] ERFE mobilizes iron stores, with *Erfe*-deficient mice exhibiting a stunted anemia recovery after hemorrhage or in states of inflammation.[57]

The erythroid form of TRF2 has emerged as akey component of the iron-sensing mechanisms involved in the feedback loop between iron availability and erythropoiesis, possibly as a modulator of the EPO sensitivity of erythroblasts.[58,59]

The expression of GDF15 is upregulated by hypoxia and iron depletion. GDF15 in turn down-modulates hepcidin values, promoting iron absorption and mobilization. It may play an important role in the iron overload associated with expansion of the erythropoietic marrow, since its serum concentrations are extremely elevated in severe thalassemias and congenital dyserythropoietic anemia type I.[60,61]

TMPRSS6 is cell membrane sensor of decreased iron availability, which suppresses hepcidin production and allows increased intestinal absorption of iron.[62] Mutations in TMPRSS6 have been associated with IRIDA,[63] and a TMPRSS6 allele associated with lower serum iron and Hb values has been identified in population studies.[64]

B. HEMATOLOGICAL MARKERS

B.1. Hb, CBC, and red cell indices

Hb is a late marker of iron deficiency, which develops in the three stages model but culminates with the appearance of anemia.WHO guidelines have selected different Hb thresholds for anemia identification based on age and other conditions:[65] for children, values of 11.0, 11.5 and 12.0 g/dL have been established based on age (6 months to under 5 years, 5 to 12 years, and 12 to under 15 years, respectively). For women and men over age 15, Hb thresholds are 12.0 and 13.0 g/Dl, respectively, while a value of 11.0 g/dL was selected for pregnant women.

The red cell indices may help identify the presence of iron deficiency erythropoiesis at a much earlier stage than Hb: patients may still have normal Hb, but present MCV and MCH which are slightly reduced or at the lower end of normal values, with increased RDW. RDW can be noticeably elevated in iron deficiency anemia, at times > 20%, reflecting the marked anisocytosis seen with iron deficient erythropoiesis.A definite reduction of MCV can be appreciated in many cases, however, only after the Hb drops below 10-11 g/dL: changes in MCV and other cell indices develop over a long time, due to the low turnover of the erythrocyte population.[66]A clear relationship connects iron supply and erythrocyte volume, not only with cell volume decreasing when iron supply decreases, but also with cell volume increasing when both iron supply and marrow erythropoiesis are increased.[6] Iron deficiency has no effect on WBC counts. PLT count is usually elevated above baseline values in iron deficiency and returns to normal with successful iron supplementation therapy. PLT counts are highest, at times above 1 million PLT/μL, in patients with iron deficiency and active bleeding. If blood is spun in a microhematocrit centrifuge, the plasma of patients with iron deficiency anemia will appear paler than usual, while it is usually much darker in thalassemia trait.

B.2. Hypochromic red cells

Iron deficient erythropoiesis causes an increase in hypochromic erythrocytes, which can be measured as the % of erythrocytes with MCHC lower than 28 g/dL (% HYPO), by certain hematology analyzers (i.e. Siemens Medical Solutions, Tarrytown, NY).[67,68] Similar parameters (Low Hemoglobin density, LHD% and %Hypo-He) are available in Beckman-Coulter and Sysmex instruments, respectively, although experience with them is more limited.[69-72] This parameter is best measured in samples less than 4 hrs. old if kept at room temperature, since storage leads to a progressive increase in hypochromic red cells because of the concomitant increase in MCV and reduction in MCHC. Refrigeration minimizes storage-induced volume changes: hypochromic red cells can be measured in samples refrigerated for up to 24 hrs. Hypochromic red cells increase with reticulocytosis, a reflection of the fact

that reticulocytes have lower MCHC than mature red cells.[4]

A classic study by Macdougall *et al.*in dialyzed patients revealed that functional iron deficiency induced by EPO treatment and the response to IV iron could be detected by changes in %HYPO.[73] Indeed, %HYPO appears to be a sensitive and early indicator of iron deficiency in a variety of studies.[74-79] Hypochromic cells were also shown to increase in normal subjects undergoing an intensive autologous donation regimen with r-HuEPO support, indicating the appearance of iron restricted erythropoiesis despite normal baseline iron status and concomitant oral iron supplementation.[80] In this study and in another, also involving normal subjects being treated with r-HuEPO,[81] iron restricted erythropoiesis was associated with a marked decrease (~75%) in serum ferritin values, indicating depletion of the readily available iron pool.

Much of the information about the %HYPO parameter has been accumulated in dialysis patients, as some guidelines have incorporated this parameter into the management of iron and erythropoietin treatment. Significantly, a European study in dialysis patients found the %HYPO to be the only independent predictor of mortality among various other iron status parameters: mortality risk increased two-fold for values > 10% compared with values <5%.[82] Patients with %HYPO > 6 are most likely to respond to IV iron therapy.[16] The1999 European Best Practice Guidelines for anemia management were tested in a clinical study that recommended a %HYPO target of < 10%: this study prospectively increased the delivered dose of IV iron to 228 dialysis patients to achieve a % HYPO < 2.5% and a serum ferritin of 200-500 ng/mL.[79] The median % HYPO decreased from 8% to 4%, median serum ferritin increased from 188 to 480 ng/mL, and median EPO dose decreased from 136 to 72 IU/kg/wk. Yet findings were mixed: this study showed the cost-effectiveness of a strategy aimed at reducing % HYPO values below 10%, although it resulted in serum ferritin values much greater than those recommended in guidelines for some of the patients. North American studies have so far failed to show usefulness of %HYPO in assessing iron availability in dialysis patients.[83,84] The reasons for the discrepancy are not yet clear. Perhaps, shipping of samples to centralized US reference laboratories may affect the stability of the %HYPO parameter. With the diminished availability of Siemens ADVIA analyzers, the commutability of %HYPO-based studies with similar parameters generated by other hematology analyzers remains to be demonstrated. A recent study suggested a cut-off value of 0.9% for %Hypo-He in predicting iron deficiency and iron deficiency anemia.[85] A new density-based cell fractionation method to quantify the presence of hypochromic red cells has shown promise as a tool to identify iron deficiency anemia in settings without access to advanced testing systems.[86]

B.3. Micro/Hypo ratioand the availability of analyzers

In the presence of a microcytic hypochromic anemia, the differential diagnosis includes iron deficiency and thalassemias, especially beta-thalassemia trait and two-gene alpha thalassemia trait.Other hemoglobinopathies with microcytosis, and congenital or acquired disorders of heme synthesis (sideroblastic anemias) should also be considered in the differential diagnosis.To aid this diagnosis, various CBC parameters have been suggested, including the Mentzer index (MCV/RBC), the Bessman index (RDW), the England and Fraser index [MCV- (5 x Hb)-RBC], the MCV/MCH ratio, the RDW/RBC ratio, and the Green and King formula [(MCV2 x RDW)/(Hb x 100).[87,88] The most useful parameter in distinguishing iron deficiency from thalassemias is the micro/hypo RBC ratio, which is usually >1.0 in thalassemia, where microcytosis prevails, and < 1.0 in iron deficiency, where hypochromia is more pronounced.[87] This parameter, however, is generated only by instruments marketed by Siemens and is not available on Coulter, Abbott, or Sysmex hematology analyzers.

B.4. Reticulocyte Hemoglobin content (CHr or RET-H*e*)

Reticulocytes spend 18-36 hrs. in the circulatory system after being released from the marrow before maturing to erythrocytes. Determination of reticulocyte cellular indices and of Hb content in particular provides a real-time assessment of the functional state of the bone marrow.[89,90] Measurements of reticulocyte Hb content,[91] (CHr or RET-H*e*, pg/cell) have been shown to identify iron deficient erythropoiesis in a variety of settings, which include pediatric and adult patients and patients treated with rHuEPO. Two studies in children have shown that CHr performs better than other traditional parameters in identifying both the presence and the future development of iron deficiency.[13,14] CHr showed greater specificity and sensitivity in assessing iron status than traditional iron parameters, after exclusion of patients with thalassemias or macrocytosis (MCV > 100 fL).[92] A reduction in CHr is the earliest indicator of functional iron deficiency: healthy subjects with normal iron stores produced a substantial fraction of hypochromic, low-CHr reticulocytes when treated with rHuEPO. Only subjects with baseline serum ferritin above 100 µg/L were able to produce normal reticulocytes.[81] Intavenous iron suppresses the production of hypochromic reticulocytes that accompanies r-HuEPO therapy.[93] In pregnant women at term, iron deficiency can be reliably identified based on either CHr and/or % HYPO.[94]

Several studies have shown the value of CHr in identifying iron deficiency in dialysis patients.[83,84,95,96] 100%

sensitivity and ~70%-80% specificity were reported in one study,[84] while other studies reported lower values.[95,96] These initial studies led to additional large clinical trials in dialysis patients, to test the role of CHr in managing the dosing of IV iron and rHuEPO. A study by Fishbane *et al*[97] randomized 157 patients to two different IV iron management strategies: one strategy was based on CHr, in which IV iron was started if CHr fell below 29 pg and one was based on traditional parameters, in which IV iron was started if ferritin fell below 100 ng/ml or TSAT below 20%. A significant reduction in exposure to IV iron was obtained in the CHr-based management, with no differences in weekly EPO dosing between the two groups.[97] Tessitore *et al*[78] compared the diagnostic power of a variety of hematological and biochemical markers in 125 dialysis patients to identify subjects with an hemoglobin response to IV iron. A combination of % HYPO >6% or CHr <29 pg showed the best diagnostic efficiency for iron deficiency (80%) based on Hb response to IV iron. Other studies have provided additional confirmation of the diagnostic value of CHr,[98,99] although one study has questioned its superiority to TSAT,[100] and only one study has shown that use of IV iron in patients with low CHr resulted in decreased weekly usage of r-HuEPO.[101]

Several studies have also validated reticulocyte Hb measurements (REF-He and Ret-Hb) generated by analyzers produced by Sysmex, which are now widely used worldwide.[70,102,103-105] Reticulocyte Hemoglobin content is decreased in the presence of alpha or beta thalassemia, while it is increased after treatment with hydroxyurea or with folate or B_{12}deficiency.

B.5. Erythrocyte ferritin

Very small amounts of ferritin are contained inside erythrocytes, in the form of two different ferritin species (alkaline and acid). They can be measured after complete lysis of the erythrocytes, and after careful removal of both plasma and contaminating WBCs, which contain approximately 1000 times more ferritin than erythrocytes. This assay is available on automated analyzers using the regular serum ferritin methodology,[106] but the process is inefficient: it requires complete removal of white cells to avoid measuring leukocyte ferritin,[107] it is insensitive to dynamic changes in iron status, and it is a rarely available diagnostic tool used by clinicians.

B.6. Erythrocyte zinc protoporphyrin

Incorporation of iron into protoporphyrin IX is the final step of heme biosynthesis: if iron is not available, Zn is inserted into the molecule to form zinc protoporphyrin (ZPP).[108] Red cell values of ZPP can be measured with a dedicated instrument called a hematofluorometer, which determines the ZPP concentration of a whole blood sample in either μm/L or as a molar ratio with hemoglobin (μmol ZPP/mol heme).[109-112] When ZPP is measured in whole blood, falsely elevated values are observed in dialysis patients, and these false values are also observed in the presence of bilirubin and various drugs. Careful washing of the red cells is required to remove these interferences, rendering this assay unsuitable for routine clinical care.[113,114] ZPP is also elevated in the presence of lead poisoning. Recent studies have shown that the diagnostic value of ZPP is inferior to that of red cell or reticulocyte parameters.[78,115] A non-invasive method for ZPP measurement in the lip has shown promise as a screening tool for iron deficiency in clinical settings without access to other resources.[116]

B.7. Peripheral Blood morphology

Red cell morphology is most informative in clear cases of iron deficient anemia, but at this stage it is solely confirmative in nature, since diagnosis can be established in many cases without looking at the peripheral blood smear. The washed-out appearance of erythrocytes and their enlarged area of central pallor are clear indicators of hypochromia. In the peripheral smear, erythrocytes show various degrees of anisocytosis, poikilocytosis, microcytosis and hypochromia, with occasional target cells, while platelets appear to be overabundant.Pencil cells and prekeratocytes are two additional abnormal red cell morphologies,which have been described in iron deficiency:[117] pencil cells are hypochromic elliptocytes, with a long axis at least three times the length of the short one, while prekeratocytes are poikilocytes exhibiting both a central pallor and one or more sub-membrane vacuoles. Pencil cells and prekeratocytes are not exclusive of iron deficiency and can also be seen in ß-thalassemia trait and the anemia of chronic disease.[118] Morphological distinction between iron deficiency and thalassemia trait is challenging: the two features considered distinctive of thalassemia minor, namely target cells and basophilic stippling, were found to be of limited usefulness in a recent study.[119]

B.8. Bone Marrow Iron determination

Iron staining of a bone marrow biopsy was regarded as a gold-standard method to assess iron stores, but this invasive, potentially painful procedure has yielded widely divergent estimates of the prevalence of iron deficiency.[120-124] The technique is based on the identification with the Prussian blue reaction (Pearl's stain) of intracellular iron deposits, which appear as round, clumped granules in macrophages (they are typically absent in iron deficiency).

A recent study in 100 non-dialysis CKD patients showed that evaluation of iron stores by iron staining of a bone marrow sternal aspirate was no better than either TSAT or ferritin in correctly identifying responders to IV iron therapy.[125] This technique also does not help doctors to identify patients at risk of developing functional iron deficiency with EPO therapy.

C. DIAGNOSTIC APPROACH TO IRON DEFICIENT STATES

Normal Hb values do not rule-out iron deficiency, especially if the prior baseline value for an individual patient is not known. Some patients may experience chronic fatigue prior to the development of anemia, although there is actually no evidence that this may be due to non-hematological effects of iron deficiency as argued by some clinicians. Iron deficiency should be suspected when Hb values over time trend downward in combination with increasing RDW and decreasing MCV/MCH. In many instances, a patient's history of chronic bleeding will prompt clinicians to add a biochemical iron study to a standard CBC. In the absence of chronic inflammatory conditions, a low or borderline-low ferritin (15-30 ng/mL) may be the only sign of the developing iron deficiency. In very young children, a careful dietary history may elicit an excessive consumption of cow's milk with a following CBC revealing an otherwise unsuspected, pronounced hypochromic microcytic anemia.

In the absence of prior laboratory encounters with an individual patient, the unexpected finding of hypochromic microcytosis with or without anemia will require that alpha or beta thalassemia traits be ruled-out. As discussed above, the micro/hypo ratio is the best parameter for this differential diagnosis. If this parameter is not available, some of the other, classic formulas may be used, although their performance is inferior to that of the micro/hypo ratio. Although informative, sTfR is not widely used and does not add significant information for simple iron deficiency cases. It should be reserved, either alone or as a ratio of sTfR/ferritin, for responding to more complex cases with associated inflammatory conditions or altered iron metabolism due to systemic disease. Since reliable hepcidin assays are only now becoming available, only limited information exists on how this parameter could be used and how much additional information it would add to serum ferritin values. It has been suggested that in the setting of ACD, the presence of significant iron deficiency component should result in lower hepcidin values.

The availability of a reticulocyte count with reticulocyte Hb content may provide an additional important tool for assessing both evolving, simple iron deficiency cases and more complicated situations like the ACD. A reduction of the reticulocyte Hb content below the lower limit of normal (usually ~ 28 pg/cell) provides direct evidence that the availability of iron to the erythropoietic marrow is limited. It may also provide a simple and reliable tool for rapid determination of the helpfulness of iron replacement therapies.

D. TREATMENT MONITORING

D.1. Oral Iron replacement therapy

Unless the patient has experienced diseases producing substantial blood loss or impaired gastrointestinal iron absorption, oral iron replacement therapy is expected to result in a measurable change in Hb values after 2-3 weeks, with Hb values approaching baseline values in 2 months, barring extremely severe cases of anemia. A widely used criterion for assessing hematological response to oral iron is a 2 gr/dl increase in Hb values after 3 weeks of therapy. Increases in absolute reticulocyte count and reticulocyte Hb content can be monitored as early as one week after initiation of oral iron therapy.[126] Therapy is usually continued for a few months after the anemia is corrected, to ensure that the storage iron is loading adequately. Monitoring both Hb values and reticulocyte parameters such as absolute reticulocyte count and reticulocyte Hb content[127] not only allows doctors to estimate the patient's response to iron replacement therapy but also to alert caregivers about either poor adherence to treatment or the presence of a concomitant pathology which could limit the effectiveness of the oral iron regimen.[5,91,128]

Biochemical parameters are less helpful to monitor therapeutic response, since plasma iron values are highly variable and may return to the normal range just because a single iron pill was taken before the blood draw. However, changes in serum iron following the administration of oral iron supplements have been used to determine iron absorption.[127] Serum ferritin is not influenced by daily variations in iron intake: thus, a progressive increase in serum ferritin (in the absence of inflammation) suggests that the patient is adhering to the therapy and that iron is effectively replenishing the body's stores. Ferritin is one of the last parameters to normalize, because it remains steadily in the normal range only after the correction of anemia, which makes it an ideal aspect to study.

D.2. Intravenous (IV) iron therapy

Reticulocyte parameters change dramatically within 24-48 hrs. following IV iron administration, with marked increases observed in the reticulocyte Hb content (CHr) as well as in reticulocyte volume and absolute reticulocyte count.[126] In normal subjects, the concomitant use of r-HuEPO and IV iron resulted in measurable increases

in the reticulocyte Hb content (CHr) and serum ferritin, with no changes in absolute reticulocyte count values compared to R-HuEPO alone.[93] These findings are consistent with the work of Finch *et al*, which shows that increased iron availability and increased erythropoiesis result in the production of larger cells with greater Hb content.[6]

In conclusion, integrating hematological and biochemical parameters allows for an earlier and more accurate diagnosis of most uncomplicated iron deficient states. In more challenging cases, accompanied by chronic inflammation or other alterations of iron handling, measurements of serum hepcidin values may provide additional useful information, with potential practical therapeutic applications as well.

Conflict of Interest: Dr. Brugnara wishes to disclose a consulting agreement with Sysmex America Inc.

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