Next-Generation Biomarkers for Iron Status

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Abstract
Iron is needed for oxygen transport, muscle activity, mitochondrial function, DNA synthesis, and sensing of hypoxia. The hierarchical master determinant of dietary iron absorption and iron distribution within the body is the peptide hormone hepcidin. Hepcidin itself is regulated by a combination of signals derived from iron stores, inflammation, and erythropoietic expansion. Iron deficiency and iron deficiency anemia are common and important conditions that can be treated with iron preparations. However, other factors besides iron deficiency can cause anemia, especially inflammation, which responds poorly to iron treatment, and inherited disorders of red blood cells, which are associated with accumulation of excess pathogenic iron. Assessment of iron status is challenging, and indices such as serum ferritin, soluble transferrin receptor, and zinc protoporphyrin have specific weaknesses. Moreover, a diagnosis of iron deficiency or iron deficiency anemia is most useful if the diagnosis also leads to effective treatment. Low levels of hepcidin allow iron absorption and effective iron incorporation into red blood cells. The best ‘biomarker’ to guide treatment may therefore be the physiological ‘determinant’ of iron utilization. Iron is also important in transplantation medicine and influences clinical outcome of arterial pulmonary hypertension; here too, biomarkers including hepcidin may be useful to actively and beneficially manage iron status.

Introduction: What Is Iron for and How Do We Regulate It?

Basic cellular physiological processes require iron; hemoglobin function, oxygen sensing, generation of energy, and maintenance of genome fidelity are iron-dependent activities. Iron can easily shuttle between its ferric and ferrous valencies, and hybridize its electron orbitals to form bonds in multiple orientations, thus
forming iron-sulfur complexes and heme, and allowing incorporation into enzymes. The utilization of iron into critical biochemical processes during Hadean/Archean time likely benefitted from the relatively reductive, oxygen-poor, acidic, and sulfur-rich environments prevalent in those epochs. However, since the Great Oxygenation Event ∼2.3 billion years ago, iron has become poorly bioavailable due to its negligible solubility at neutral pH. Therefore, iron is now both indispensable for life but challenging to assimilate, and, due to its reactivity, toxic in excess.

In humans, requirements for iron are not constant or stable over time and vary with age (growth rate), pregnancy, and even altitude, and availability of iron is dependent on the amount and type of nutrition. Humans maintain iron homeostasis by controlling iron absorption, with iron excretion being almost unregulated [1]. An average of about 1 mg dietary Fe is taken up per day, but twenty times that amount is recycled by macrophage-mediated degradation of hemoglobin from senescent erythrocytes. Hepcidin is the 25-amino-acid peptide hormone secreted from the liver, which controls both iron recycling and absorption from the diet. Hepcidin achieves this by inhibiting the function of the iron exporter protein, ferroportin (fig. 1) [2]. Hepcidin therefore controls both the total amount of iron and its partitioning within the body. The molecular action of hepcidin and its regulation (and the relationship of hepcidin to mechanisms that maintain cellular iron homeostasis) are the subject of many reviews [1, 3, 4].

Hepcidin synthesis is controlled by three major inputs. First, accumulation of iron in serum or in the liver is sensed and leads to increased synthesis of hepcidin, which blocks iron absorption by enterocytes and macrophage recycling, returning the system to equilibrium. Genetic lesions that cause low levels of hepcidin underlie iron-overloading disorders such as hereditary hemochromatosis. Second, hepcidin synthesis is also switched on by inflammation. High levels of hepcidin cause low levels of serum iron, which may be critically protective against infection by iron-requiring blood-dwelling micro-organisms that could cause fatal sepsis. Third, because the major single requirement for iron in the body is for erythropoiesis, the loss of blood causes profound hepcidin suppression that frees up available iron stores and enhances iron absorption, to supply the bone marrow and facilitate rapid replacement of lost erythrocytes. A mediator of hepcidin suppression in this context is the recently identified erythroblast-secreted protein erythroferrone [5]. Erythropoietin stimulates erythroferrone production, and erythroferrone acts directly on the liver to suppress hepcidin. The iron overloading observed in some inherited red cell disorders (for example thalassemia intermedia) may be caused by high levels of erythroferrone secreted by erythropoietin-stimulated erythroblasts, leading to persistently suppressed
hepcidin. Thus, hepcidin regulation incorporates signals from iron levels (liver stores and serum iron), from inflammation, and from erythropoietic demand. The signals are all believed to act at the level of the hepcidin promoter through the binding of transcription factors that are activated by the different physiological inputs, and which together control the transcription of the gene. Other levels and mediators of hepcidin regulation are known or suspected to contribute to hepcidin synthesis, but are not as well characterized as the signals discussed above and are omitted for reasons of space.

**Fig. 1.** Hepcidin and the iron cycle. Around 1 mg per day of heme and nonheme iron is absorbed by enterocytes in duodenal villi and transferred to serum where it is bound by transferrin – each transferrin protein can bind up to two atoms of iron. Transferrin delivers iron to tissues and cells expressing transferrin receptors; 60% of the body’s transferrin receptors are in the bone marrow, where the iron is incorporated into heme in the hemoglobin of developing red blood cells (RBC). Senescent red blood cells are recognized and phagocytosed by splenic and liver macrophages. Erythrocytes are degraded, and iron is released from heme by the enzyme heme oxygenase 1. The liberated iron can either be stored within macrophages as ferritin or released back into serum. Around 20 mg of iron per day is recycled in this way. Loss of iron is not well regulated and, except in situations of blood loss including menstruation, it amounts to approximately 1 mg per day. Both the final step of iron transfer from enterocytes into plasma and the final step of iron recycling back into plasma through macrophages are mediated by a multitransmembrane protein ferroportin, the only known mammalian iron exporter. Ferroportin activity is negatively regulated by the circulating peptide hormone hepcidin that is synthesized primarily by the liver. Changes in the amount of hepcidin therefore regulate iron absorption by the intestine and iron efflux by macrophages and so determine the total amount of iron in the body and its distribution between serum and tissues.
Why Do We Need to Know the Iron Status?

Because of the importance of iron for physiology, iron deficiency is associated with an array of health impairments [6], especially anemia but also suboptimal cognitive, psychomotor, and physical development, although much of the data underlying associations with developmental outcomes in infants are based on observational analyses [7]. Nevertheless, iron deficiency (usually defined using serum ferritin concentration cutoffs) is highly prevalent in infants and pregnant women in the developing world, and is thought to account for half the anemia that is present in one quarter of the world’s population [8]. The socioeconomic burden of anemia is considerable, and combating anemia is a major aim of global health programs. Anemia is also highly prevalent amongst hospitalized patients in the developed world, but as discussed below, the cause of this anemia is often inflammatory rather than due to iron deficiency. Iron status has been correlated with outcome of kidney [9] and liver transplantation [10], and heart disease [11], and iron availability can influence response to hypoxic pulmonary hypertension [12], heart failure [13], as well as recovery from iron deficiency anemia. In summary, iron status is relevant for a variety of important disorders.

Why Is It Important to Guide Iron Therapy?

A variety of methods to increase iron availability are available, including iron tablets, iron-containing multiple micronutrient powders, iron fortification of foods, and iron preparations for intravenous delivery. Population-level iron supplementation is recommended in areas of high anemia prevalence, but evidence has emerged and is still accumulating that universal nontargeted oral iron treatments can have significant downsides.

First, several trials of different types of iron supplementation in different territories have indicated that iron may exacerbate the incidence and/or severity of infectious diseases, notably malaria, but also potentially respiratory infections [14, 15]. The underlying cause may be that iron deficiency is relatively protective against pathogen growth (especially in the context of malaria [16]) and that in some individuals the potential hematological benefit of iron is outweighed by the risk of developing an infection. This is a complex question and the evidence is not entirely clear or indeed consistent, but nevertheless in areas of high infectious burden and suboptimal access to health care, the infection risk of iron supplementation is a significant consideration.

A related but different issue is the effect of iron supplementation on gut microbiota. Iron excess has been shown to cause the outgrowth of potentially
pathogenic gut flora at the expense of non-iron-requiring and protective lactobacilli [17]. Diarrhea is observed in populations given oral iron supplements, and this along with impaired food absorption capacity and increased intestinal inflammation, caused by imbalanced gut flora, is obviously an undesirable consequence of iron therapy. Reducing the dose of iron may theoretically in part address this problem. Intravenous iron is also available and has demonstrated beneficial profiles of hematological response, but again is associated with an increased incidence of infection [18], and its use in community populations may be compromised by financial and other considerations.

Anemia can have many causes that are not iron related, but iron therapy is very likely to be most effective as a treatment for iron deficiency anemia. An important example is the anemia of inflammation that is the most common cause of anemia in hospitalized patients. In this setting, anemia is due to the effect of inflammatory cytokines that can impair erythropoiesis and cause persistently high levels of hepcidin. The elevated hepcidin chronically downregulates the capacity for iron absorption and prevents release of iron into serum [19]. As such, anemia of inflammation is often poorly responsive to iron, and patients with this condition given iron may be more at risk of developing gastrointestinal problems due to iron being retained in the intestinal lumen. In contrast, hepcidin levels are typically low in iron deficiency, allowing utilization of dietary iron supplements. This contrast illustrates the general proposal that appropriate targeting of iron therapy is required to minimize risks and maximize benefits. Not all types of anemia are caused by iron deficiency and not all will respond to iron; iron is not harmless but is a potential cause of intestinal dysfunction and may exacerbate infections.

Is Hepcidin the Best Index to Guide Iron Therapy?

Ideally, iron should be given to individuals that are both iron deficient and able to efficiently absorb and utilize iron in the form in which it is provided. Conversely, iron could and perhaps should be withheld from individuals who are iron replete or who have inflammation. Finding biomarkers not only to diagnose iron deficiency but, equally important, also to identify the ability to absorb and utilize iron is challenging. Serum ferritin has been a key index for iron status for decades but is limited for at least two reasons. First, although low ferritin is generally associated with low iron stores and hence iron deficiency, ferritin is also an acute-phase protein and transient inflammation increases its levels, thus masking its regulation by iron. Second, ferritin is not directly influenced by erythropoietic demand for iron. In a cohort of African children of mixed iron, hemoglobin, and inflammatory status, ferritin levels were similar in anemic and
nonanemic children, whereas hepcidin was lower in anemic children, likely reflecting the sensitivity of hepcidin to the iron requirement of the bone marrow [20].

Of other indices used to assess iron deficiency, soluble transferrin receptor is an indicator of erythropoietic drive, but lacks specificity for iron deficiency and is increased by hemolytic anemia and sickle cell disease; moreover, different assays give different results. Zinc protoporphyrin levels increase when there is a lack of iron to incorporate into heme, but this increase can be caused by iron deficiency or anemia of inflammation or thalassemia. Transferrin saturation is decreased by iron deficiency but also by the hypoferricemic response to inflammation. Hepcidin is the only single index that incorporates sensitivity (at the transcriptional level, as described above) to iron levels, inflammation, and erythropoietic demand for iron. The log ferritin/soluble transferrin receptor index may also reflect a balance of these three inputs and appears to be a useful biomarker although again its utility may be affected by the lack of standardized soluble transferrin receptor assays as well as the need for calculation.

Serum hepcidin measurement also lacks standardization although this may be achievable to some extent by the use of adjustment factors to allow comparison between assay systems. Hepcidin possesses a further advantage over the other indices above, which may be illustrated by considering the rare autosomal recessive condition of iron-refractory iron deficiency anemia (IRIDA). Refractory iron deficiency anemia (that does not respond to oral iron) is not uncommon but is usually caused by Helicobacter pylori infection, autoimmune gastritis, or celiac disease [21]. However, for a small proportion of patients, a different cause has been identified. These patients have very low serum ferritin, low transferrin saturation, increased soluble transferrin receptor levels, and high zinc protoporphyrin as well as anemia, and generally no evidence of inflammation. These indices diagnose iron deficiency anemia, but this diagnosis does not lead to successful treatment. The cause of IRIDA is mutation of a gene (TMPRSS6) that usually restrains synthesis of hepcidin [22, 23]. In affected patients, hepcidin levels are increased, despite iron deficiency, and this increase explains both the etiology of the anemia and the resistance to oral iron therapy. By measuring the determinant of iron absorption and iron trafficking, more insight can be gained than by measuring other iron status indices. Although genetic IRIDA is rare, the concept illustrated is that, in general, hepcidin may most accurately report the ability to utilize iron (as we found in a study of African children of mixed-cause anemia [24]). Treatment of IRIDA is usually by intravenous iron, but even the response to this is suboptimal and short lived because iron is retained in macrophages. Antagonists of hepcidin are being developed and may have a critical role to play in IRIDA and perhaps in anemia of inflammation.
We recently demonstrated the ability of hepcidin as a single index to identify iron deficiency in a large population of African children; sex, age, wasting, and carriage of hemoglobinopathy did not significantly affect this diagnostic property of hepcidin [20]. Furthermore and importantly, we found that hepcidin measurement could also be used to categorize anemia into likely iron-responsive and non-iron-responsive types [20]. This concept is not synonymous with traditional definitions of iron deficiency, iron deficiency anemia, and anemia of inflammation, but rather hepcidin measurement may reflect the combined need for iron and the ability to respond to oral iron.

In individuals, anemia is likely to be due to several causes of greater or lesser contribution; tissue iron deficiency may often exist alongside moderate inflammation and erythropoietic iron demand. Attempting to understand the balance of these signals is challenging and essentially has led to altered cutoffs for serum ferritin in the presence of inflammation, and the development of the log ferritin/soluble transferrin receptor index, for example. However, the binding of different transcription factors to the hepcidin promoter also represents these same signals, and the output of the balance (increased or decreased hepcidin) appears to be the major determinant of iron absorption and utilization. In other words, determining iron status has been a difficult and evolving empirical exercise that unconsciously attempted to measure the signals that we now know control hepcidin synthesis (table 1). For this reason, hepcidin itself is likely to be an excellent guide for iron supplementation and could be used to target oral iron treatments more effectively.

What Are the Barriers to Implementing Screen-and-Treat Programs?

The above proposal is supported by theoretical considerations, animal model work, our work in African children, and work of others in different settings [25, 26]. However, not all analyses have led to the same conclusion [27] and it will be important to define those populations, subgroups, and disease settings where hepcidin performs best. A critical part of this is to ensure that the hepcidin assay used is able to detect low concentrations of serum hepcidin; some recent analyses have been to some extent compromised by a limit of detection that excluded many (if not most) samples in the study. Standardization of the assay will also be important in this regard.

Definitive evidence of the ability of hepcidin to guide iron supplementation is still lacking, although prospective trials are underway to assess this issue. Should they be successful, the rationale for hepcidin-guided iron supplementation (screen-and-treat programs) will be better supported, but implementation will also depend on the availability of affordable and reliable assays that work
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rapidly enough to inform iron treatment decisions before an individual’s iron status significantly alters. One possibility may be a dipstick measurement, the development of which would not appear to present an insurmountable technological difficulty.

Are There Other Emerging Candidates for Iron Biomarkers?

The recently discovered erythroblast-derived suppressor of hepcidin, erythroferrone [5], may have some value as a biomarker, although this concept is necessarily largely theoretical at present, as the role of erythroferrone in humans has not been confirmed and assays to measure erythroferrone in human sera are not currently validated. Nevertheless, it is possible that erythroferrone could indicate the degree of erythropoietic response to recombinant erythropoietin (for example in the treatment of renal failure) and erythroferrone/hepcidin ratios could inform as to the extent of bone marrow iron demand in thalassemia, and potentially the degree of bone marrow suppression in anemia of inflammation.

Table 1. Equivalence of indices used to assess the iron status and known molecular regulators of hepcidin

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<thead>
<tr>
<th>Molecular regulators of hepcidin synthesis</th>
<th>Biomarkers used to assess the iron status</th>
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<td>signal regulating hepcidin (direction of regulation)</td>
<td>index of the iron status</td>
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<tr>
<td>BMP6/SMAD signaling (↑)</td>
<td>Liver iron stores</td>
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<tr>
<td>HFE/TfR2, SMAD signaling (↑)</td>
<td>Iron available in blood</td>
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<tr>
<td>IL-6/STA3 signaling (↑)</td>
<td>Inflammation</td>
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<tr>
<td>Erythroferrone (↓)</td>
<td>Erythropoietin signaling in bone marrow following blood loss or in thalassemia</td>
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Hepcidin is upregulated by iron: both by iron in the liver, which drives hepcidin transcription through bone morphogenetic protein 6 (BMP6) and SMAD signaling, and by iron in serum bound to transferrin, again via SMAD transcription factors but also requiring HFE and TfR2. Inflammation stimulates hepcidin synthesis through cytokines, especially IL-6 (although IL-22 and type-I interferon may contribute), and the transcription factor STAT3. Bone marrow demand for iron is communicated to the liver via the erythroblast-secreted hormone erythroferrone, which causes suppression of hepcidin. Commonly used indices of iron status (e.g. C-reactive protein, which is used to assess inflammation but influences the interpretation of serum ferritin concentrations) show a very close relationship with the known factors that control hepcidin, so that attempts to ascertain iron status using a combination of these indices could be viewed as measures of the balance of hepcidin regulation.
What About Iron and Hepcidin in Nonhematological Conditions?

This paper has focused mainly on iron status and hepcidin in relation to anemia, but evidence from the past few years has demonstrated associations of iron and hepcidin in the contexts of kidney disease, transplantation medicine, heart failure, and response to hypoxia. There is a great deal of interest in the role of hepcidin in renal medicine, for example on whether inflammation-induced hepcidin contributes to anemia, the use of hepcidin as a marker to predict responsiveness to erythropoietin [28], as a corollary of glomerular filtration rate, and as a potential therapeutic target [29]. Hepcidin may also be of use after transplantation to monitor renal function and iron status, as iron overload is believed to be harmful following transplantation [9]. Interestingly, the reverse may be the case in liver transplantation, as high hepatic hepcidin and low transferrin receptor expression predict successful weaning from immunosuppression and tolerance to the hepatic graft [10].

Preoperative anemia is associated with higher postoperative mortality in heart surgery patients, and a major cause of this anemia appears to be functional iron deficiency (in which total iron stores may not be low, but iron is relatively unavailable for erythropoiesis) [11]. Interestingly, a recent study found that of all iron and hematopoietic indices tested, only (high) hepcidin was an independent indicator of mortality [30]. Hepcidin controls iron availability not only to the bone marrow but to cells in general – and iron is required both for muscle function and for appropriate sensing of hypoxia, both of which are likely to be important in the context of heart surgery. This finding may also be of relevance to a previous observation that intravenous iron carboxymaltose improved the functional status, symptoms, and the quality of life in patients with heart failure and iron deficiency [13]. Thus, although further prospective trials are needed, hepcidin may be of use to identify at-risk groups and guide iron therapy in the area of cardiac disease.

Concluding Remarks

Measuring iron status is difficult, as several factors need to be incorporated and balanced appropriately: iron stores, inflammation, serum iron levels, and bone marrow demand for iron. However, the regulation of hepcidin expression represents a molecular integration of these inputs, and the levels of hepcidin then determine at least in part the likely efficacy of iron therapy. The issue is important not only because of the prevalence of iron deficiency and anemia worldwide and their contribution as comorbidities to conditions such as heart disease and kidney failure, but also because iron therapy is increasingly recognized as being
in some circumstances actively harmful. Therefore, targeting of iron will be important. The use of hepcidin as a guide for iron therapy is currently a justifiable concept, but a great deal more evidence in different disease conditions and settings is required, and more standardized assays may need to become available before its potential can be realized.

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