HEPCIDIN IN THE DIAGNOSIS OF IRON DISORDERS

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Abstract
The discovery of the iron-regulatory hormone hepcidin in 2001 has revolutionized our understanding of iron disorders, and its measurement should advance diagnosis/treatment of these conditions. Although several assays have been developed, a gold standard is still lacking, and efforts toward harmonization are ongoing. Nevertheless, promising applications can be already glimpsed, ranging from the use of hepcidin levels for diagnosing iron-refractory iron deficiency anemia to global health applications such as guiding safe iron supplementation in developing countries with high infection burden.

Pathophysiological background
Hepcidin, a liver-derived peptide hormone, is a key regulator of systemic iron homeostasis, and its unbalanced production contributes to the pathogenesis of a spectrum of iron disorders. Hepcidin functions by blocking iron flows into plasma: duodenal absorption, release from macrophages recycling old red blood cells (RBCs), and mobilization of stored iron from hepatocytes (for extensive reviews see 1,2). This is achieved by hepcidin causing degradation of its receptor, the iron transporter ferroportin. Hepcidin production is tightly regulated: i) it is increased by plasma and liver iron as a feedback mechanism to maintain stable body iron levels; ii) it is decreased by erythroid activity to ensure iron supply for erythropoiesis; and iii) it is increased by inflammation as a host defense mechanism to limit extracellular iron availability to microbes 1-3. Because hepcidin levels reflect integration of multiple key signals involved in iron regulation, and hepcidin directly controls iron absorption and bioavailability in circulation 4-6, its measurement should be a useful clinical tool for the management of iron disorders. Although a thorough understanding of its unique advantages over traditional biomarkers of iron status in different conditions will require larger studies with head-to-head comparisons, we discuss below some studies already supporting the distinct utility of hepcidin measurements.

Hepcidin structure and kinetics
The bioactive circulating form of hepcidin is 25 amino acids in size. N-terminal degradation leads to smaller isoforms (hepcidin-24, 23, 22 and 20) of unknown significance 7-9. These isoforms are generally present in diseases with elevated
hepcidin-25 levels, including chronic kidney disease and sepsis\textsuperscript{10,11}. Circulating hepcidin is bound to $\alpha$-2 macroglobulin and albumin, but estimates of this binding vary from <3\% to 89\% of total\textsuperscript{12,13}. The impact of binding on hepcidin activity or assay performance is unclear. Hepcidin is rapidly excreted by the kidney and reabsorbed in the proximal tubules by megalin-dependent endocytosis, but urinary hepcidin levels generally correlate with serum levels\textsuperscript{14,15}, with the exception of renal diseases\textsuperscript{16}. Nevertheless, urinary hepcidin measurements may have utility in specific conditions such as noninvasive measurement in children, screening in low-resource settings, or prediction of acute kidney injury\textsuperscript{17}.

**Pre-analytical, analytical and post-analytical aspects (table 1a)**

The development of assays to quantify hepcidin in biological samples has proven challenging. Since hepcidin is a small, evolutionary conserved peptide, it is difficult to generate antibodies for laboratory assays. Hepcidin quantification is further complicated by its tendency to aggregate and stick to laboratory plastics\textsuperscript{18}. Nonetheless, a number of well-performing assays have been established, whose list is provided in Supplementary Table 1. They are divided into two major groups: mass spectrometry-based (MS) and classical immuno-assays (IA)\textsuperscript{19}. MS assays require relatively expensive equipment, but are able to distinguish the hepcidin isoforms. IA assays generally lack specificity for hepcidin-25 and measure total hepcidin levels. The relevance of specifically measuring hepcidin-25 instead of total hepcidin for clinical decision-making, however, is unclear.

In the absence of primary reference material, a reference method, and a commutable calibrator, absolute hepcidin levels differ widely between assays (up to 10-fold)\textsuperscript{20}. Studies aiming at harmonization are ongoing\textsuperscript{21}, but for now these differences preclude data comparability and the establishment of a universal reference range. Rather, each method/laboratory needs to establish rigorous age- and gender-specific reference ranges, preferably not only for hepcidin, but also for hepcidin/transferrin saturation and hepcidin/serum ferritin ratios. To date, only two large studies in Holland ($n=2,998$)\textsuperscript{22} and Italy ($n=1,577$)\textsuperscript{23}, have evaluated serum hepcidin variations at population level, clearly showing that hepcidin levels are lower in pre-menopausal than post-menopausal women, and are highly correlated with serum ferritin levels. In smaller studies, the within-subject variation of serum hepcidin was relatively high: hepcidin increased with prolonged fasting\textsuperscript{24}, and showed both circadian rhythm and
considerable (27-50%) day-to-day variation. Hepcidin-25 values decrease within 1-2 days with storage at room temperature, but are stable at 4, -20 and -80 °C for at least a week, 4 weeks and ~2 years, respectively.

General considerations for the clinical applications of hepcidin measurement
Like every hormone, hepcidin is under the influence of many different stimuli. Figure 1 summarizes the opposing effects exerted by a number of physiological and pathological conditions. Of note, the response is often quite rapid, making circulating hepcidin a very dynamic compartment. For example, hepcidin production increases substantially within few hours after iron administration or inflammatory stimulation. Since many of the conditions listed in Figure 1 are common, several stimuli may be present simultaneously, with hepcidin output depending on the relative strength of each. For example, in severe iron deficiency (ID) hepcidin production tends to be low even in the presence of inflammation. Similarly, in conditions of ineffective/expanded erythropoiesis like in non-transfusion dependent thalassemias (NTDT), signals released by bone marrow erythroid precursors tend to override those from replete iron stores. This results in relative hepcidin suppression in NTDT, other iron-loading anemias, and even in β-thalassemia trait. One such erythroid signal, erythroferrone, has been recently identified in a mouse model. In line with this pathophysiological model, serum hepcidin fluctuates in transfusion-dependent β-thalassemia, with levels increasing soon after RBCs transfusions (which suppress ineffective erythropoiesis) and declining during inter-transfusional periods.

Because of the highly dynamic and multifactorial regulation, a key practical message is that, in a given individual, the correct interpretation of hepcidin levels requires accurate knowledge of the overall clinical context.

Most promising applications of hepcidin measurements in hematology (table 1b).

**Diagnosis of Iron-Refractory Iron Deficiency Anemia (IRIDA).** This genetic disease, due to mutations in the hepcidin inhibitor TMPRSS6 (encoding Matriptase-2) is characterized by iron deficiency anemia with inappropriately normal or high hepcidin levels. Thus, oral iron is ineffective and parenteral administration is needed to achieve at least a partial response. In classical IDA, hepcidin is
suppressed below the limit of detection in biological fluids. By contrast, detection of pseudo-normal/elevated serum hepcidin in an appropriate clinical context can be considered virtually diagnostic of IRIDA, even without confirmation by \textit{TMPRSS6} sequencing, i.e. in a young patient with unexplained IDA not responding to oral iron and with positive family history. Measurement of hepcidin may also help in the diagnosis of other atypical microcytic anemias due to rare genetic disorders of iron metabolism or heme synthesis (reviewed in \textsuperscript{42}), although data are presently insufficient.

\textbf{Diagnosis of Iron Overload (IO) Disorders}. Classical type-1 (\textit{HFE}-related) hereditary hemochromatosis (HH) is by far the commonest genetic disorder leading to IO in populations of Northern European descent \textsuperscript{43}, but in other ethnicities “atypical” IO accounts for up to 35-40\% of cases \textsuperscript{44}. In type-1 HH at diagnosis, serum hepcidin levels are typically in the low-normal range, and inappropriate for the degree of iron overload (manifesting as a low hepcidin/ferritin ratio) \textsuperscript{45}. Hepcidin is further suppressed after normalization of iron stores by phlebotomies \textsuperscript{15,45}, and shows a blunted response to oral iron \textsuperscript{29}. This is also seen in type-3 HH (\textit{TFR2}-related) \textsuperscript{29}, while in types-2A/B or “juvenile” hemochromatosis (\textit{HJV}- or \textit{HAMP}-related, respectively) hepcidin levels are markedly suppressed/undetectable \textsuperscript{19,46}. By contrast, in HH type-4B or atypical ferroportin disease due to mutations in \textit{SLC40A1} conferring partial or complete resistance to hepcidin \textsuperscript{47}, serum hepcidin levels are substantially increased \textsuperscript{48}. Some data support using hepcidin measurement as a guide for the follow-up genetic testing of IO patients, particularly in populations where classic HFE-HH is rare \textsuperscript{49}. Furthermore, during treatment of type-1 HH, monitoring hepcidin to prevent its complete suppression may curb iron hyperabsorption in the maintenance phase, possibly decreasing the need for phlebotomies. In iron-loading anemias, hepcidin measurement may also be valuable for identifying the most severely affected patients, helping to predict the development of IO and guiding the therapy.

\textbf{Diagnosis and management of iron deficiency anemia (IDA)}. In IDA, hepcidin levels are generally suppressed to allow maximal iron absorption \textsuperscript{15,19}, but some patients, particularly the elderly \textsuperscript{50}, may have detectable hepcidin levels because of co-morbidities like renal, inflammatory, or neoplastic diseases. Since hepcidin directly controls iron absorption \textsuperscript{4,5}, serum hormone levels have the potential to predict poor
responsiveness to oral iron, preventing possible detrimental effects of oral iron on the gut microbiome and metabolome, and eliminating delays before switching to intravenous iron. The usefulness of measuring basal hepcidin to personalize the optimal route of iron administration has been recently showed in patients with IDA, chronic rheumatic anemia, and chemotherapy-associated anemia. However, large prospective trials are needed to confirm this attractive hypothesis. Hepcidin has also been proven effective for detecting ID in blood donors, the prevention of which is a major concern for blood service.

Notably, determination of serum hepcidin may help to solve a major global health problem, i.e. the appropriateness of oral iron supplementation in children from regions with high infection burden. ID is highly prevalent in these regions, and affect children's physical growth and cognitive performance. Nonetheless, systematic iron supplementation has been associated with serious adverse outcomes, including increased mortality. Such detrimental effects have been mainly attributed to increased vulnerability to malaria and microbial agents, which are engaged in the host-pathogen battle for essential iron. Hepcidin capability to integrate competing signals (anemia, iron deficiency, and infection), makes its measurement promising in this setting. Indeed, two recent elegant studies using iron isotopes showed that low serum hepcidin was a good predictor of erythrocyte iron incorporation in African children, and suggest hepcidin as a guide for distinguishing individuals “ready to receive iron” from those in whom it should be avoided. This fascinating hypothesis, requiring the development of a validated point-of-care hepcidin assay, is actively under evaluation. However, since iron-induced reticulocytosis could increase susceptibility to malaria, and low hepcidin values do not necessarily exclude a concurrent infection, safe iron supplementation in malaria endemic areas should imply not only the ability to absorb and incorporate iron, but also effective measures to prevent exacerbations of infections.

**Distinction between iron deficiency (ID) and anemia of chronic disease (ACD).**

ACD is a common condition with complex pathogenesis. Hepcidin-driven iron maldistribution plays a substantial role as iron is trapped in macrophages and is less available for erythropoiesis. Patients with chronic inflammatory disorders are also at risk of developing ID. For example, occult/overt blood loss frequently occurs in conditions like inflammatory bowel diseases, chronic hemodialysis, and concomitant
use of NSAIDs or antithrombotic drugs. In such setting, ID is difficult to detect using traditional iron biomarkers, but hepcidin may be helpful. Patients with inflammatory disorders and concomitant ID typically have lower hepcidin levels as compared to those with “pure” ACD. Indeed, hepcidin has been proven effective in distinguishing IDA from ACD in patients with rheumatoid arthritis, inflammatory bowel diseases, cancer-related anemia, critical illnesses, and also in African children. This, again, can help in personalizing iron therapy to avoid delays or unnecessary/harmful treatment. However, because of the highly variable cut-off reported with different assays, harmonization of hepcidin assays is necessary before giving practical recommendations.

**Companion diagnostic for novel therapies.** Hepcidin discovery has opened unprecedented therapeutic opportunities for iron disorders. Hepcidin pharmacology is an active field with a number of drugs in the pipeline, including hepcidin agonists for treatment of IO, and hepcidin antagonists for treatment of iron restriction in ACD (reviewed in). Measuring hepcidin levels is anticipated to be helpful both in selecting patients and monitoring the effects of novel targeted therapies.

Supplementary Table 2 shows examples of the diagnostic performance of hepcidin (e.g. AUC$_{ROC}$, sensitivity, and specificity) in different clinical settings.

**Conclusions**
Hepcidin is a promising tool to be added to the present battery of diagnostic tests for iron status, especially in ID, where it has the potential to differentiate from ACD, and to inform about the ability to respond to oral iron. However, full inclusion in clinical practice and public health requires further efforts to harmonize the assays, to assess the relevance of measuring specific hepcidin isoforms, to define clinical decision limits, and to make validated assays universally available.

**Authorship**
**Contribution:** D.G., E.N., and D.W.S. conceived and co-wrote the manuscript.

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measurements to the medical, scientific and pharmaceutical communities via the www.hepcidinanalysis.com initiative at a fee for service basis. E.N. is consultant and stock holder for Intrinsic LifeSciences, Merganser Biotech and Silarus Therapeutics.

**Figure Legend.**

**Figure 1**: Clinical conditions known to influence circulating hepcidin levels.

**Abbreviations**: GFR, glomerular filtration rate; CKD, chronic kidney disease; TMPRSS6 (transmembrane protease serine 6), the gene encoding for matriptase-2; IDA, iron deficiency anemia; HH, hereditary hemochromatosis; HCV, Hepatitis C Virus.

**Figure references**: CKD 11,16; Red Blood Cell transfusions 36; iron administration 29,30; replete iron stores 1; TMPRSS6 variants 40,71; infections/inflammatory disorders 27,28,53; ineffective erythropoiesis 3,36, hypoxia 72,73; Erythropoietic Stimulating Agents 74; chronic liver diseases 75; alcohol abuse 76; HCV 77; “hemochromatosis mutations” 1,29,45,46; sexual hormones, both testosterone 78 and estrogens 79,80.

**References**


Table 1. Hepcidin measurement in clinical practice. A Decalogue for the hematologists.

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<tr>
<th>A) Checklist before ordering the assay</th>
<th>Comments</th>
<th>References</th>
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<tr>
<td>1. Ensure local availability of a validated assay</td>
<td>See text and Supplementary Table 1</td>
<td>(19)</td>
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<td>2. Ensure control of pre-analytical conditions (including diurnal rhythm)</td>
<td>See text</td>
<td>(8, 25, 26)</td>
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<td>3. Refer to age- and sex-specific ranges</td>
<td>Significant differences between males and females, particularly during fertile period.</td>
<td>(22, 23)</td>
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<td>4. Interpret hepcidin value into a minimum laboratory context (CBC, ferritin, transferrin saturation, CRP, serum creatinine, liver function tests).</td>
<td>See Figure 1</td>
<td>-</td>
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<td>5. Be aware of many potential confounders/comorbidities in the individual patient</td>
<td>See Figure 1</td>
<td>-</td>
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<th>B) Most promising applications</th>
<th>Virtually diagnostic in an appropriate clinical context</th>
<th>(41, 42)</th>
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<td>6. Evaluation of suspected IRIDA</td>
<td>e.g.: ferroportin disease due to hepcidin resistant mutations (see text)</td>
<td>(34, 37, 45, 46, 48, 49)</td>
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<td>8. Diagnosis of concomitant iron deficiency in patients with anemia of chronic disease</td>
<td>e.g.: selection of patients for direct I.V. supplementation; oral administration in children from developing countries with high prevalence of infectious diseases (see text)</td>
<td>(6, 50, 52-54, 62)</td>
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<td>9. Guide for iron therapy</td>
<td>To be confirmed by further studies</td>
<td>(69)</td>
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CBC= complete blood count; CRP= C-reactive protein; IRIDA= Iron-Refractory Iron Deficiency Anemia.
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