Hepcidin as a Potential Biomarker for the Diagnosis of Anemia

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ABSTRACT

There are several blood-based markers to assess iron stores, but they all have some limitations. Hepcidin, a low-molecular-weight peptide hormone, is produced mainly by the liver. It is the main regulator of iron homeostasis by preventing iron release into plasma from absorptive enterocytes and macrophages. This review aims to critically assess existing data on potential role of hepcidin in diagnosis, particularly the (pre) analytical implications of the hepcidin measurement. There is a well-known causative correlation between hepcidin and iron deficiency. Therefore, hepcidin is considered as a promising marker in the assessment of iron status, particularly in patients with a diagnostic dilemma, such as patients with chronic renal disease and infants. The clinical implications of this peptide hormone in diagnosis of other diseases have been expanded in the recent studies, including elevated hepcidin levels in neoplastic diseases, sepsis, and inflammation. The potential role of hepcidin in diagnosis is controversial in the various types of iron deficiency because data are conflicting (as in anaemia of chronic disease) or limited (as in infants), whereas in the case of hereditary haemochromatosis, it has been proposed that hepcidin may be used for stratification of molecular testing, or to improve the frequency of phlebotomy, however, this issue still needs to be investigated. Due to lack of a clinically approved test, the medical application of this peptide as a biomarker in diagnosis is restricted. Recently, assays have been developed to determine hepcidin levels in serum and urine, facilitating the future use of hepcidin in research and clinical practice.

Key words: Hepcidin, iron deficiency, anemia, peptide hormone

INTRODUCTION

Biologically important hemoproteins such as hemoglobin (Hb) and myoglobin contain iron as an essential element. Therefore, iron homeostasis is critical for normal erythropoiesis and other cellular processes.1 As there is no regulatory mechanism for excreting iron from the body apart from losses due to menstruation and other bleeding processes, dietary iron uptake and status of internal iron stores are the major regulators of constant iron balance.2 Anemia represents a worldwide public health problem with symptoms caused either by nutrient deficiency to hereditary changes in absorption and metabolism of iron.3 Although different forms of enzymes and proteins have been involved in iron transporting, it was found that iron homeostasis is tightly controlled via a hepatic-produced hormone, i.e. hepcidin, by tightly affecting the production of erythrocytes. Therefore, discovery of hepcidin in 2001 changed our understanding of iron problems and its measurement should help with diagnosis and treatment.4 High levels of hepcidin inhibit intestinal absorption and macrophage recycling of iron, resulting in iron-restricted erythropoiesis and anemia. In contrast, low levels of hepcidin stimulate the supply of iron to the bone marrow for Hb synthesis and production of erythrocytes.5 Erythropoiesis, after erythropoietin therapy or hemorrhage, inhibits hepcidin by decreasing transferrin saturation and releasing of erythroblast and the hepcidin inhibitor (erythroferrone). However, decreased erythropoiesis, reducing iron consumption, increasing transferrin saturation and stimulating hepcidin transcription.6 Hepcidin acts by binding with a ferroprotein, a multipass membrane protein and thus inhibiting the iron efflux into the plasma.7 Many studies have confirmed the relationship between hepcidin production and pathogenesis of iron-linked diseases.8-10 Elevated hepcidin levels may be associated with anemia unresponsive to treatment with iron, while decreased plasma hepcidin may result in excess iron in the circulation with tissue-iron deposition.10-11 Additionally,
higher hepcidin levels have been involved in many clinical conditions, such as inflammation, neoplastic diseases, and sepsis. In future, the dependence on proven detection methods will enhance the possibility of hepcidin use as a novel biomarker in clinical practice.

**Hepcidin synthesis and regulation**

Hepcidin is a small cationic peptide of 25 amino acids and mass of 2.7 kDa. Because of its antifungal and antibacterial activities, hepcidin was first identified as a hepatic produced antimicrobial proteins. Then, the antimicrobial activity and origin of hepcidin were found in the liver, reflecting its new official nomenclature. Although the major source of hepcidin synthesis is in the liver, recent findings also revealed extra-hepatic synthesis of hepcidin in the kidney, heart, retina, alveolar cells, bacteria-activated neutrophils, macrophages, pancreatic β-cells, and adipocytes. However, substantially lower expression of hepcidin gene has been observed in these tissues compared to with hepatic production and its relative importance remains unknown.

Hepcidin peptide in humans is encoded by the *hepcidin antimicrobial peptide* gene that present in chromosome 19. Hepcidin is encoded as a preprohepcidin protein, a 84 amino acid precursor, which is subsequently converted to the bioactive hepcidin peptide by two sequential cleavages in the cytoplasm of hepatocyte and in the blood. After hepatic production, hepcidin reaches blood circulation and provides widespread in the tissues. All hepcidin isoforms 20, 22, and 25 are excreted in urine. Hepcidin-20 and 22 are regarded as degradation byproducts of hepcidin.

As indicated by nuclear magnetic resonance (NMR) spectroscopy studies, human hepcidin's molecular structure consists of one beta sheet in the peptide backbone, which has an eight cysteine loop formed by four disulfide bonding molecules. It is considered necessary to perform out biological activities with cysteine and N-terminal amino acids, which form the copper and nickel binding (ATCUN) motif. Hepcidin works in three primary iron-metabolism target cell types, namely, enterocytes, hepatocytes, and reticuloendothelial macrophages, which are considered the three primary plasma iron sources (transferrin-Fe2+). Hb incorporated in the erythrocytes is attributed to approximately 66% iron in the human body. Macrophages ingest senescent erythrocytes, then releasing iron in blood or storing it as ferritin. Similarly, intestinal epithelial cells absorb all heme and non-heme iron, thus resulting in iron released in plasma or iron stored as ferritin. Endocytosis is used in cells expressing transferrin receptors to get transferrin from the bloodstream. This iron is used for erythropoiesis by bone marrow erythroid cells, while it is used for storage by liver cells. Ferroportin is the iron exporter across membranes responsible for iron efflux from tissue to the circulation for use in erythropoiesis. Hepcidin binds with ferroportin and induces an adverse effect on erythropoiesis by stimulating the uptake and then degradation of ferroportin.

**Pathogenesis of iron metabolism disorders**

Iron deficiency diseases are the first type of iron metabolism disease. Iron deficiency conditions are associated with reduction of ferritin, reduced serum iron and transferrin saturation, microcytosis, and hypochromia. However, in certain cases, like newborns, inflammatory diseases, or severe renal disorders using erythropoietin-stimulant medications, the diagnosis of iron deficiency diseases depending on these laboratory results is difficult. Therefore, hepcidin may be an important biomarker in these situations.

**Hepcidin in clinical diagnosis**

Plasma levels of hepcidin are well correlated with plasma ferritin in healthy persons. Moreover, serum hepcidin concentrations are significantly reduced in iron deficit patients. Hepcidin is an acute phase protein of type II, with elevated hepcidin correlating with higher serum ferritin in severe inflammations. In comparison with traditional biomarkers, hepcidin with ferritin has significant value in diagnosis of different forms of iron diseases. In addition to importance of ferritin as a reliable biomarker for iron storage, the pathogenesis of many iron conditions like anemia of chronic disease (ACD) or haemochromatosis includes variations in hepcidin levels. Thus, hepcidin determination may provide an indicator on etiology of iron diseases and enable diagnosis before depletion of iron stores. The diagnostic role of hepcidin in situations that have diagnostic difficulties with traditional biomarkers is outlined below.

**Hepcidin in infants with iron deficiency anemia (IDA)**

Infants with iron-deficient erythropoiesis or latent iron deficiency had difficulty in diagnosis with the conventional iron laboratory parameters. In these infants, ferritin is not an ideal indicator of anemia so that transferrin levels are often low in infants. Additionally, there is no diagnostic benefit for serum transferrin receptor (sTfR). To avoid permanent changes of neurodevelopment, early diagnosis of iron deficiency in children is necessary. The content of reticulocyte Hb (Chr) represented the availability of iron for incorporation in the bone marrow just a few days earlier. The best iron indicator for infants seems to be Chr, but there is still limited availability of this test. Even before anemia occurs, hepcidin has been recommended to diagnose children with iron deficiency. Genetic diseases rarely result in IDA in children. Iron-refractory IDA (IRIDA) is a genetic disease marked by inherited hypochromic microcytic anemia, typically unresponsive to oral intake of iron, and partially improves with intravenous intake of iron. In IRIDA patients, mutations are heterogeneous, including splicing, frame-shift, nonsense and missense mutations in TMPRSS6, an encoding gene for matriptase-2. Normally, matriptase-2 suppresses the expression of hepcidin. Mutation in this gene contributes to improperly elevated hepcidin levels in humans and animals, making intestinal absorption of iron impossible. Early diagnosis of iron deficiency diseases is impossible with the traditional biomarkers and is required to use the appropriate iron therapy (parenteral). Therefore, hepcidin quantification can provide a diagnostic method for IRIDA to direct genetic testing.
Hepcidin can identify iron deficiency before the development of anemia develops, generating a potential identification method in infants. Nevertheless, no trials have yet evaluated the application of hepcidin. Moreover, trials in infants revealed no correlation between prohepcidin and iron status.27-29 Since infants cannot control intestinal absorption of iron, it is likely that it would not have any diagnostic benefit for hepcidin because hepcidin is the regulator of iron absorption.30 Trials must explain why IRIDA does not respond to oral therapy of iron. Without inflammation, IRIDA may be suspected in patients with iron deficiency and elevated serum hepcidin. Moreover, another study demonstrated that in elderly anemic individuals with concomitant inflammation, hepcidin is not better than ferritin in the diagnosis of iron deficiency. In these patients, hepcidin has a significant positive association with ferritin and C-reactive protein.31 Unfortunately, no trials have examined hepcidin in the diagnosis of iron deficiency.

**Hepcidin in anemia of chronic diseases with or without iron deficiency anemia**

Higher levels of serum hepcidin are crucial for pathogenesis of ACD. The ACD is identified by cytokines-induced macrophage iron retention, which is partly mediated by elevated serum hepcidin, triggered by interleukin-6.26,32 ACD is associated with normochromic normocytic anemia and reduced reticulocyte numbers, accompanied by erythropoiesis inhibition.32 It is difficult to diagnose iron deficiency in ACD patients because coexisting blood loss drug intake or inborn defect in Hb synthesis could affect them. Concerning to treatment, it is critical to differentiate between ACD with iron deficiency and ACD, where iron therapy is essential in the first case, but controversial and not currently recommended in the second.32 Ferritin and other biochemical markers of iron are influenced by acute phase reaction. Therefore, it is difficult to distinguish between ACDs with or without iron deficiency depending on serum ferritin. In both cases, reduced iron level and low saturation of transferrin are recognized. ACD patients with IDA often have microcytes with severe anemia compared to those with ACD alone.32 The sTfR is, newer marker, a truncated portion of the membrane receptor, which increases in deficiency of iron available for erythropoiesis. The sTfR/ ferritin ratio could help differentiate ACDs with or without iron deficiency. However, this ratio is not commonly used in clinical practice because of the lack of standardization of the sTfR assays.21 Hepcidin is controlled by inflammation and by iron in the body.33 Hepcidin levels may be able to distinguish between ACDs with or without iron deficiency depending on the predominant regulatory mechanism. Interestingly, increased production of cytokines like interferon gamma by lymphocytes and interleukin1-β and 6 and tumor necrosis factor-alpha by macrophages reduces synthesis of endogenous erythropoietin, inhibits the erythropoiesis process, enhances serum levels of hepcidin, and, hence, activates erythropagocytosis.34

Serum hepcidin levels are considerably lower in iron deficiency patients compared with healthy subjects.17,35 Hepcidin is increases by inflammation in ACD.26 Hepcidin levels in urine have shown to respond markedly better in acute phases after injection of lipopolysaccharide in healthy persons than serum ferritin.19 Hepcidin also may be more likely to be more susceptible to iron imbalances than ferritin or other biomarkers. This remains completely default. Because hepcidin is involved in the acute phase reaction, the usage of hepcidin measurement is uncertain. Just few clinical trials have examined whether hepcidin levels have become a valuable biomarker for iron deficiency diagnosis in ACD patients with or without IDA. In initial trials, the predominant regulator for the hepcidin was deficient iron stores because serum hepcidin was low in IDA, irrespective of inflammation, and hence, the possibility for a hepcidin cut-off could be established to distinguish ACD with or without IDA.26,37,38 Nevertheless, even ferritin differs considerably within the ACD subgroups with or without iron deficiency, rendering it difficult to understand a possible benefit additional to hepcidin compared to ferritin in inflammation.26 Till now, there have been no trials establishing a definite limit for the IDA diagnosis. A study by Lasocki et al.37 identified an iron deficiency cut-off in patients with intensive care. Just 5 of 51 patients suffered from iron deficiency (just 3 with concurrent inflammation). Recently, studies revealed the difficulty in distinguishing between ACD with and without iron deficiency depending on the serum hepcidin. Thomas et al.39 identified latent IDA, ID, ACD, and ACD with IDA in anemic patients, depending on ferritin index plot. ACD was not distinguished from ACD/IDA by hepcidin only. Nevertheless, Thomas et al.39 showed that combining hepcidin and CHe instead of ferritin index plot because hepcidin could react to the hematologic state quicker than ferritin. Therefore, it is important to remember that the only rapid reaction of hepcidin seen to date is a rapid rise in the urine due to inflammation.19 As a result, more studies must establish the variation in hepcidin among ACD, IDA and ACD with IDA. Moreover, the importance of hepcidin measurement as a diagnostic marker needs to be evaluated in patients with unclear IDA.

One proposed approach to predicting classical IRIDA is to normalize hepcidin to other iron markers such as the ratio of transferrin saturation/log hepcidin or transferrin saturation/log ferritin.40 Another study revealed that most severe IRIDA patients are biallelic TMPRSS6 mutations and, when unrecognized, the second allele may be genetically obscure.41 Generally, people with two allele mutations have a more severe phenotype and are less responsive to iron therapy than those with one allele mutation.40 Interestingly, many TMPRSS6 single-nucleotide polymorphisms have been detected in blood donors to increase susceptibility to iron deficiency.42

**Hepcidin and iron deficiency in chronic renal disorders**

Erythropoietin-stimulating agents (ESA) produce a substantial rise in erythropoietic activity in chronic renal disorder, which is followed by a sharp increase in demand for bioavailable iron. Even with adequate storage of iron in the body and intake of oral iron therapy, ESA can produce a reduction in iron levels and transferrin desaturation, leading to iron deficient erythropoiesis.21 This represents the third obstacle in the
diagnosis of iron deficiency. Diagnosis of this disease requires the appropriate dose of ESA or intravenous iron for optimal response. Iron deficiency is predicted by low ferritin, but ferritin is unpredictable because of common inflammation-induced effects.\textsuperscript{43} The saturation of transferrin reduces with inflammation and varies with serum iron changes daily. Moreover, after several weeks after parenteral iron, it is not interpretable (100% saturation). In patients with inflammation, elevated ferritin and -20 percent transferrin saturation, functional iron deficiency may be suspected. Although transferrin saturation and ferritin are the major parameters in the diagnosis of iron deficiency, trials to find a better index continues.\textsuperscript{44,45} The percentage of red hypochromic cells will recognize developing IDA, but for changes in this marker, it takes long periods of iron deficient erythropoiesis. Reticulocyte number and Hb alteration can distinguish patients as “responding” or “unresponders”. CHr appears to be a promising predictor of ESA dosage.\textsuperscript{21,46} Therefore, it appears worth to investigating the additional importance of hepcidin in this situation.

Hepcidin has been recognized as a major determinant in the pathogenesis of anemia in patients with chronic kidney disease (CKD), and explains the disturbed metabolism of iron and ESA resistance.\textsuperscript{47} Hence, hepcidin may be an effective predictor of response and guidance for ESA or parenteral iron therapy. Most patients with an ineffective ESA response have functional iron deficiency, which cannot be detected by saturation of transferrin or ferritin.\textsuperscript{48} These patients are hypothesized to have higher levels of hepcidin, thus expected to be hyporesponsive and require higher doses of ESA. In CKD and hemodialysis, elevated hepcidin levels were identified.\textsuperscript{49-51} Nevertheless, not only anemia or iron can affect hepcidin concentrations in CKD, but several other factors like hypoxia, inflammation and erythropoietin (endogenous or exogenous).\textsuperscript{52} Furthermore, hepcidin is negatively correlated with glomerular filtration rate (GFR), contributing to elevated hepcidin levels due to a reduction in renal excretion.\textsuperscript{49} Peters et al.\textsuperscript{52} showed no considerable association between hepcidin-25 and eGFR. However, the same study revealed that hepcidin-25, hepcidin-20, and -22 isoforms had an inverse correlation with eGFR. In the control group, hepcidin-22 was less than the measurable value, but detectable in patients with CKD and hemodialysis. With the exception of hepcidin-25, hepcidin-20, and -22 accumulated in renal failure. However, these findings need to be verified by more trials. Another study showed that dialysis can clear hepcidin, but it increased to pre-dialysis levels before the next dialysis.\textsuperscript{53}

Clinical trials evaluating the effect of hepcidin in differentiating responder and hypo-responder to ESA are few. One study revealed no variation in the intensities of hepcidin levels between erythropoietin responsive and hypo-responsive patients.\textsuperscript{54} In dialysis patient, hepcidin is associated with anemia and is compatible with a possible role for elevated hepcidin levels in renal anemia.\textsuperscript{49} However, in the same study revealed an inverse correlation between hepcidin levels and the dose of erythropoietin, which reduces the effect of hepcidin in predicting a higher need for erythropoietin. Another study revealed that unresponsive patients have reduced serum hepcidin, but these findings may be biased because unresponsive patients obtained significantly higher doses of erythropoietin than responsive patients.\textsuperscript{55} Swinkels and Wetzels\textsuperscript{56} recommend observing the initial hepcidin changes after a first dose of parenteral iron and/or erythropoietin because a single dose will predict patient response before starting the anemia therapy. However, further trials must elucidate the potential role of hepcidin in diagnosis.

**Hepcidin and iron overload**

Iron-storage disorders are the second group of disorders of iron metabolism. Nevertheless, high ferritin concentrations do not have the lack of specificity to distinguish between genetic cases with a continuous deposition of iron and moderate iron overload due to disorders that affect the liver (for example; viral hepatitis, alcoholic or non-alcoholic fatty hepatic disorder).\textsuperscript{57,58} The pathogenic factor in iron deposition of hereditary haemochromatosis (HH) type 1 (HFE hemochromatosis), the main cause of hereditary iron overload, appears to be a reduced hepcidin production from the liver.\textsuperscript{59} Thus, the HFE C282Y homozygous induces decreased hepcidin expression, leading to the deposition of parenchymal iron. Mutations of other genes involved in hepcidin activity, synthesis, or regulation induce less common types of HH.\textsuperscript{59} A continuous erythropoiesis decreases hepcidin in anemia associated with iron overload due to inactive erythropoiesis like thalassemia, which contributes to an overload of iron in this disorder. As a result, produced a severe reduction in hepcidin levels in types treated with a non-blood transfusion method. Whereas, hepcidin levels are higher in patients severely treated with blood transfusion than those treated with non-blood methods, because of elevated iron in the body and reduced erythropoiesis.\textsuperscript{60}

It is unclear if a distinguishing role of hepcidin presents in cases of hyperferritinaemia. When there is a family index case, the final diagnosis of HH depends on familial screening.\textsuperscript{58} Phlebotomy is the treatment for HH. Therapy is controlled by ferritin measurement since ferritin is correlated with iron levels in the liver and is a sensitive indicator of cirrhosis, albeit with the same non-specificity difficulty. Ferritin level <50 mg/L is characterized as a consensus therapeutic target without relevant proof of its true significance.\textsuperscript{61}

The diagnosis is based on genotyping of HH. Hepcidin can be used as a direct measure for genetic iron overload, since hepcidin levels (or hepcidin to ferritin ratio) are reduced in HH, but elevated in ferroportin disorders.\textsuperscript{62} Clinical characteristics like Hb (which is reduced in secondary iron overload and ferroportine disorder), family history (genetic disorder), concurrent disorder (such as, hepatitis and alcoholic) and age are used for the rational targeting of genes. To stratify genetic testing, hepcidin may then be applied.\textsuperscript{59} Therapeutically, hepcidin has a more significant use in HFE-haemochromatosis, specifically to determine need for phlebotomy. Firstly, hepcidin appears unsuitable to this objective, since abnormal levels of hepcidin trigger the disorder and are not sensitive to stores of iron. Actually, in spite of continuous phlebotomy, a persistent decrease in hepcidin results in a persistent increase in intestinal absorption of iron. Nevertheless, low hepcidin
concentrations in serum and urine much further reduced with iron loss by phlebotomy in HFE-related HH patients, and this reduction in hepcidin associated with a reduction in transferrin saturation.\textsuperscript{52,63} These findings indicate that a phlebotomy “overshot” may have an adverse effect by further reducing hepcidin, with subsequent increase in intestinal absorption of iron. Another study proposed an upward review of the existing ferritin target (less than 50 µg/L to look for the optimum balance of reduced ferritin and a minimum reduction of hepcidin.\textsuperscript{62} Therefore, hepcidin assessments should be determined in further clinical studies. It is not obvious if the targets of phlebotomy may be identified by hepcidin levels or by the hepcidin to ferritin ratio. There are no studies available related to treatment monitoring in another HH mutation. However, hepcidin is a possible therapeutic aim in haemochromatosis, which may considerably improve the treatment and follow-up of HH patients, rendering the possible diagnostic utility of hepcidin unclear.

Recently, the association between hepcidin and cancer-related anemia has been studied. This study showed that anemia is associated with hepcidin overexpression in patients with increased C-reactive protein, suggesting that hepcidin plays a distinct role in the development of cancer-related anemia.\textsuperscript{64}

\textbf{Hepcidin assays}

Developing assays to estimate hepcidin quantity in biological fluids is challenging. It is difficult to develop antibodies for laboratory tests because hepcidin is an evolutionarily conserved small peptide, which tends to aggregate and adhere to laboratory plastics.\textsuperscript{65} However, two major assays have been developed; mass spectrometry and classical immunoassays.\textsuperscript{66} Although mass spectrometry-based tests are more costly, they can differentiate between hepcidin isoforms. Immunoassays often lack the specificity of hepcidin-25 and detect total levels of hepcidin. However, the importance of measuring hepcidin-25 specifically rather than total hepcidin for clinical decision-making is debatable.

Absolute hepcidin levels vary considerably (up to 10 fold) between tests in the absence of a main reference material, a reference technique, and a commutable calibrator.\textsuperscript{67} While harmonization research is ongoing, these discrepancies now prevent the possibility of comparing the data and establishing a uniform reference range.\textsuperscript{68} Instead, each method/lab should develop strict reference ranges for age and sex, for hepcidin to serum ferritin and hepcidin to transferrin saturation ratios, in addition to that for hepcidin. Till now, only four population studies, comprising of two in Italy (n: 1577, n: 1391),\textsuperscript{69,70} one in the Netherlands (n: 2998),\textsuperscript{71} and one in West Africa (n: 1316),\textsuperscript{72} have estimated differences in serum hepcidin in the general population, clearly indicating that hepcidin levels increased considerably as the number of metabolic syndrome characteristics increased.\textsuperscript{70} Moreover, hepcidin levels are higher in postmenopausal versus premenopausal women and are highly correlated with serum levels of ferritin.\textsuperscript{69,71} Furthermore, hepcidin and hepcidin to ferritin ratio were not correlated with atherosclerosis or with cardiovascular disease.\textsuperscript{73} Whereas findings from smaller studies revealed that the within-subject variability of hepcidin levels was relatively high. Additionally, hepcidin levels were higher in prolonged fasting,\textsuperscript{74} and revealed both circadian rhythm and significant variation (27-50%) from day to day.\textsuperscript{75} Values of hepcidin-25 reduce within 1-2 days with storage at room temperature but they remain stable for 1 week, 4 weeks, and 2 years at 4°C, -20°C, and -80°C, respectively.\textsuperscript{76,77}

\textbf{CONCLUSION}

Although its exciting findings at the beginning, the data about the diagnostic utilization of hepcidin remain limited. There have been some advances in measurement of hepcidin-25 quantitatively, but several pre-analytical issues still unanswered. Recently, trials have focused on hepcidin agonists and antagonists based on their possible therapeutic applications. Now, the question: Will hepcidin can be used in diagnosis in the future?

\textbf{Ethics}

\textbf{Peer-review:} Externally peer-reviewed.

\textbf{Authorship Contributions}

Concept: Z.H.F., J.A.M., Design: Z.H.F., J.A.M., Data Collection or Processing: Z.H.F., J.A.M., Z.M.Y., S.M.M., Analysis or Interpretation: Z.H.F., Literature Search: Z.H.F., J.A.M., Z.M.Y., S.M.M., Writing: Z.H.F.

\textbf{Conflict of Interest:} No conflict of interest was declared by the authors.

\textbf{Financial Disclosure:} The authors declared that this study received no financial support.

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FATHI et al. Hepcidin as a Biomarker for Anemia