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Hepcidin in Human Iron Disorders: Diagnostic Implications

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BACKGROUND: The peptide hormone hepcidin plays a central role in regulating dietary iron absorption and body iron distribution. Many human diseases are associated with alterations in hepcidin concentrations. The measurement of hepcidin in biological fluids is therefore a promising tool in the diagnosis and management of medical conditions in which iron metabolism is affected.

CONTENT: We describe hepcidin structure, kinetics, function, and regulation. We moreover explore the therapeutic potential for modulating hepcidin expression and the diagnostic potential for hepcidin measurements in clinical practice.

SUMMARY: Cell-culture, animal, and human studies have shown that hepcidin is predominantly synthesized by hepatocytes, where its expression is regulated by body iron status, erythropoietic activity, oxygen tension, and inflammatory cytokines. Hepcidin lowers serum iron concentrations by counteracting the function of ferroportin, a major cellular iron exporter present in the membrane of macrophages, hepatocytes, and the basolateral site of enterocytes. Hepcidin is detected in biologic fluids as a 25 amino acid isoform, hepcidin-25, and 2 smaller forms, i.e., hepcidin-22 and −20; however, only hepcidin-25 has been shown to participate in the regulation of iron metabolism. Reliable assays to measure hepcidin in blood and urine by use of immunochemical and mass spectrometry methods have been developed. Results of proof-of-principle studies have highlighted hepcidin as a promising diagnostic tool and therapeutic target for iron disorders. However, before hepcidin measurements can be used in routine clinical practice, efforts will be required to assess the relevance of hepcidin isoform measurements, to harmonize the different assays, to define clinical decision limits, and to increase assay availability for clinical laboratories.

Iron is required for the function of oxygen-binding molecules, mainly hemoglobin and myoglobin, and iron-containing enzymes, including the cytochrome system in mitochondria. Cellular iron is mostly bound within iron protoporphyrin (heme) and iron–sulfur clusters, which serve as enzyme cofactors, or stored within the core of ferritin multimers. Without adequate iron, cells lose their capacity for electron transport and energy metabolism. However, the redox activity of iron can also cause damage, primarily by the production of reactive oxygen radicals. As such, iron concentrations must be tightly regulated both at the cellular and the systemic level. A crucial element in the maintenance of systemic iron homeostasis is effective communication between cells that absorb iron from the diet (duodenal enterocytes), use iron (mainly erythroid precursors), and store iron (hepatocytes and tissue macrophages). The peptide hormone hepcidin interacts with the cellular iron exporter ferroportin and thus is now recognized as the key regulator of systemic iron homeostasis. Here, we review the major advances from studies on the structure, kinetics, regulation, and function of hepcidin and the current state-of-the-art in hepcidin assays. We furthermore summarize human studies of hepcidin in physiological and pathophysiological states, and discuss the evidence of the therapeutic potential of modulating hepcidin concentrations. We conclude by highlighting the most promising applications of hepcidin assays in diagnostic medicine.

Hepcidin Synthesis and Structure

Human hepcidin is predominantly produced by hepatocytes as a 25 amino acid peptide (2789.4 Da) (1, 2), that is secreted into the circulation. Subsequent aminoterminal processing of the 25 amino acid form can result in the appearance of 2 smaller hepcidin forms of 22 and 20 amino acids. These hepcidin peptides form a hairpin structure, with 4 intramolecular disulfide bridges (Fig. 1) (3). On the basis of some studies, investigators have reported that hepcidin binds divalent...
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Metals (Cu²⁺, Fe²⁺, Zn²⁺, and Ni²⁺), but these findings are inconsistent, e.g., some studies have suggested the presence of iron in the core of the peptide in a tetrahedral sulfur coordination (4–6), whereas others provide evidence for an amino-terminal Cu(2⁺)–Ni(2⁺)-binding (ATCUN) motif (7, 8). The ability of hepcidin to bind iron and other divalent metals suggests there may be a nonhormonal role for hepcidin in iron metabolism or a conformational mechanism for uptake of divalent metals as part of hepcidin’s hormonal role in regulating ferroportin degradation.

Much is still unknown on the origin of the smaller isoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas isoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that calcium-independent tissue activity in pancreas exoisoforms of hepcidin, although data suggest that 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**Fig. 1. Molecular structure of hepcidin.**
A model of the structure of hepcidin-25 according to Jordan et al. (3) is shown, with the β-sheets (grey arrows), the peptide backbone (grey), the disulfide bonds (1–8, 2–4, 3–6, and 5–7) (yellow), positively charged arginine and lysine residues (blue), and the negatively charged aspartic acid residue (red).

Hepcidin Kinetics

Circulating hepcidin was recently found to be bound to α₂-macroglobulin with relatively high affinity and to albumin with relatively low affinity. On the basis of theoretical calculations, 11% of hepcidin was estimated to be freely circulating (28). Whether binding to these carrier molecules influences the functional properties of hepcidin is uncertain.

Hepcidin clearance is assumed to occur via cellular codegradation with ferroportin at its sites of action, and via excretion by the kidneys. Because of its low molecular weight and small radius, unbound hepcidin is likely to freely pass into the glomerular filtrate. In small studies in humans the fractional excretion of hepcidin has been calculated to be as low as 0%–5% (29, 30), either because it is reabsorbed, similarly to other small peptides, or because it is not freely filtered. Evidence for the latter explanation comes from the finding of increases of only 1- to 6-fold of serum hepcidin concentrations in patients with glomerular dysfunction (15, 16, 29, 31, 32) compared with the 20- to 30-fold increase of serum β₂-microglobulin. The excretion of the latter low molecular weight protein is known to be almost completely governed by glomerular filtration. It is possible that binding to α₂-macroglobulin or other carrier proteins prevents circulating hepcidin from being freely filtered. Alternatively, the otherwise expected increased circulating concen-

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**Note:**

DMT, divalent metal transporter; IL, interleukin; C/EBP, CCAAT-enhancer-binding protein; c, competitive; MS, mass spectrometry; IDA, iron deficiency anemia; HH, hereditary hemochromatosis; HD, hemodialysis.
trations in patients with decreased renal filtration may be offset by a compensatory feedback decrease in hepatic hepcidin production.

It is speculative, but conceivable, that under certain conditions hepcidin escapes renal tubular reabsorption. This lack of reabsorption may play a role in several disorders of iron metabolism that are associated with tubular dysfunction and increased concentrations of urine hepcidin, such as inflammation, iron overload, and malaria (33, 34). Possible local tubular production of hepcidin must also be taken into account in the interpretation of urine as a mirror of serum hepcidin concentrations in such studies (25) as well as possible defective tubular reabsorption of hepcidin. Additional studies to investigate the possibility that local urinary infections or tubular dysfunction can contribute to hepcidinuria are warranted.

Hepcidin Function

Hepcidin-25 is thought to be the major regulator of dietary iron absorption and cellular iron release. It exerts its regulatory function by counteracting the function of ferroportin, the major cellular iron exporter in the membrane of macrophages, hepatocytes, and the basolateral site of enterocytes. Hepcidin-25 induces the internalization and degradation of ferroportin (35–38), resulting in increased intracellular iron stores, decreased dietary iron absorption, and decreased circulating iron concentrations (Fig. 3).

In addition to its role in regulating systemic iron metabolism, hepcidin may also contribute to host defense. Hepcidin was originally identified as an antimicrobial peptide (1, 11). Although in vitro studies suggest bacteriocidal effects of hepcidin, these effects would require concentrations higher than those observed in the circulation. Such concentrations may be achieved locally, for instance in phagosomes of infected macrophages (39). Hepcidin might also contribute indirectly to host defense by reducing plasma iron concentrations. Iron is necessary for microbial growth, and reductions in plasma iron are bacteriostatic. Moreover, hepcidin was found to modulate lipopolysaccharide-induced transcription in both cultured macrophages and in vivo mouse models (40). This latter observation suggests a role for hepcidin in modulating acute inflammatory responses to bacterial infection.

Hepcidin produced by various cell types other than hepatocytes (see above) may have local effects in these tissues. Through an autocrine interaction with ferroportin, local hepcidin may protect the nearby cells from iron deficiency, prevent extracellular oxidative stress, affect inflammatory responses, and/or deplete extracellular iron pools that are available for extracellular pathogens (27, 40–42). Although the smaller forms of hepcidin do not elicit a hypoferremic response, it is currently unknown whether they retain other identified biological functions of hepcidin-25 (e.g., in host defense or in metal binding) (4, 6, 11, 43).
Several physiologic and pathologic processes regulate the synthesis of hepcidin (Fig. 3) [reviewed in (44)]. Situations in which demand for circulating iron is increased (particularly erythropoietic activity) elicit a decrease in hepatocellular hepcidin synthesis. These conditions include iron deficiency, hypoxia, anemia, and conditions characterized by increased erythropoietic activity. A decrease in hepcidin results in the release of stored iron and an increase in dietary iron absorption. On the other hand, infection and inflammation cause an increase in hepcidin synthesis. This increased synthesis leads to a deficiency of iron available for erythropoiesis, and is considered to be the mechanism underlying reticuloendothelial (RE) iron sequestration, intestinal iron absorption impairment, and low serum iron concentrations characteristic of anemia of chronic disease.

The functional signaling routes by which (a) iron status, (b) erythropoietic activity, (c) hypoxia, and (d) inflammation affect hepcidin expression are increasingly being investigated. These routes comprise 4 highly interconnected regulatory pathways (Fig. 4).

**Hepcidin Regulation**

Liver iron stores and circulating transferrin-bound iron (Tf-Fe₂) each provide distinct signals to influence hepatocyte hepcidin expression (44–46) and are considered separately (Fig. 4). Circulating transferrin ap-
pears to be sensed via a hepatocellular complex, which includes transferrin receptor-1 (TfR1), TfR2, and hemochromatosis iron protein (HFE). Defects in TfR2 and HFE lead to decreased hepcidin concentrations via the extracellular signal-regulated kinases: the mitogen-activated protein kinase (ERK/MAPK) pathway and/or the bone morphogenetic protein/mothers against decapentaplegic homolog (*Drosophila*) (BMP/SMAD) pathway (Fig. 4). Intracellular iron stores communicate with hepcidin via BMPs, particularly BMP-6, in a paracrine or autocrine fashion. These extracellular signaling molecules act on hepatocellular BMP receptors to activate the intracellular SMAD signaling pathway and increase hepcidin transcription. Hemojuvelin (HJV), a BMP coreceptor (47), is crucial for hepcidin expression because various hepcidin regulatory pathways converge at this membrane-bound protein. Under low iron conditions, membrane-bound HJV is...
Cleaved by matriptase-2, a transmembrane protease serine 6, encoded by the transmembrane protease, serine 6 (TMPRSS6) gene, expressed predominantly in the liver (48, 49). This cleavage by matriptase-2 weakens the BMP signaling.

REGULATION BY ERYTHROPOIETIC SIGNALS
Administration of erythropoiesis-stimulating agents (ESA) was found to decrease (hepatocyte) hepcidin production in murine, human, and in vitro studies (50–53). Erythropoiesis requires considerable amounts of iron, so suppression of hepatic hepcidin synthesis by erythropoietic signals is of great physiological importance. However, how erythropoiesis regulates hepcidin is not clear yet. The hypothesis that erythropoietin (EPO) acts directly on hepatocyte receptors in cell culture (51) could not be confirmed in animal models for anemia, which showed that decreased hepcidin expression depends on erythropoiesis and is not directly mediated by EPO (53, 54).

Recent observations suggest that the erythropoietic signal may include 1 or more proteins released at sites of active erythropoiesis, i.e., growth differentiation factor-15 (growth differentiation factor-15 (GDF15) and twisted gastrulation protein homolog-1 (TWSG1). These molecules, like BMPs are members of the transforming growth factor-β family and thus possibly act through effects on the BMP/SMAD pathway (Fig. 4) (55–57). Correlations between the expression of TWSG1 expression and serum iron parameters and serum hepcidin concentrations have not yet been determined in humans. Neither of these factors, however, appears to be required to mediate the decrease in hepcidin observed with EPO administration. It is likely that additional erythropoietic factors downregulating hepcidin expression will be identified.

REGULATION BY HYPOXIA
Decreased hepcidin expression has been reported in response to hypoxia in vivo (58, 59). This effect might be attributable in part to the effect of hypoxia on EPO expression and thus erythropoietic activity and/or possibly via a direct interaction with hepatocyte receptors (51). In addition, the lower hepcidin concentrations observed in response to hypoxia may be attributed to liver-specific stabilization of hypoxia-inducible factor (HIF)-1 (60), with downstream effects on the BMP/SMAD signaling pathway (61, 62). Whether HIFs directly bind to the hepcidin promoter is currently controversial. However, there are indirect mechanisms by which HIFs may regulate hepcidin expression. Increased HIF activity is associated with increased matriptase-mediated cleavage hemojuvelin and thus decreased hepcidin expression (62). Moreover, the systemic iron regulation by the hepcidin-ferroportin axis acts in harmony with the enterocyte cellular iron homeostasis control system. Mice lacking intestinal HIF2α have decreased expression of both ferroportin and divalent metal transporter 1, the principal iron importer at the apical surface of duodenal enterocytes, and thus fail to induce iron absorption even upon lowering of hepcidin expression (63). These data suggest that in hypoxic (or iron deficient) conditions, expression of HIF2α promotes iron absorption from the intestine via increased activity of divalent metal transporter 1 and ferroportin. This result implies that HIF2α-induced changes in iron transport can override the effects of the hepcidin-ferroportin regulatory axis.

REGULATION BY INFLAMMATION
The increase in hepcidin expression with inflammation is primarily mediated by interleukin-6 (IL-6). The hepatocellular interaction of IL-6 with its receptor activates the janus kinase/signal transducer and activator of transcription-3 signaling pathway (59, 64–66). Hepcidin expression is also increased by oxidative or endoplasmic reticulum stress. This stress response can be controlled by the transcription factor C/EBP-response-element-binding-protein-H (67) or by the stress-inducible transcription factors CCAAT-enhancer-binding protein (C/EBPα) and C/EBP-homologous protein (68). Alcohol-dependent down-regulation of C/EBPα, probably through radical oxygen species, might be responsible for a decrease of hepcidin expression and subsequent (mild) iron overload in heavy drinkers (69, 70).

INTERACTION BETWEEN HEPcidIN REGULATORY PATHWAYS
The activation of the hepcidin regulatory pathways described above are all dependent on interactions of circulating or autocrine factors with hepatocyte membrane sensors to control the hepatic production of hepcidin for proper maintenance of systemic iron homeostasis [reviewed in (44)]. There are multiple potential opportunities for cross communication among these pathways, and experimental evidence shows that these pathways do not function completely independently of each other.

Serum hepcidin concentrations appear to be determined by the relative strengths of the individual regulators. In an attempt to delineate relative strengths of these regulators in patients with iron metabolism disorders, we constructed an algorithm to predict relative hepcidin concentrations based on certain serum pa-

6 Human genes: TMPRSS6, transmembrane protease, serine 6; HFE, hemochromatosis; PER1, period homolog 1 (Drosophila); TIMELESS, timeless homolog (Drosophila); CLOCK, clock homolog (mouse); HAMP, hepcidin antimicrobial peptide; TFR2, transferrin receptor 2; HFE2, hemochromatosis type 2 (juvenile).
The quantification of hepcidin has been found to be complicated by its tendency to aggregate (79) and to stick to laboratory plastics, necessitating implementation of robust laboratory procedures. Furthermore, progress in developing a conventional immunochemical hepcidin assay has been hampered by difficulties in generating specific antihepcidin antibodies in hosts such as rabbits. This difficulty is due to the small and compact structure of hepcidin, which leaves scarce antigenic epitopes (Fig. 1), and the high degree of conservation of hepcidin among a wide range of species, which diminishes the elicitation of an immune response in host animals (50).

Interpretation of hepcidin test results might be influenced by whether the various assays are detecting freely circulating hepcidin, hepcidin bound to a carrier protein, or both of these hepcidin species. Lastly, the presence of hepcidin-22 and -20 isoforms, which play no role in the regulation of iron metabolism, can interfere with the quantification of hepcidin-25 in immunoassays that use antibodies that react with all of these hepcidin isoforms.

FIRST-GENERATION (SEMIQUANTITATIVE) HEPCIDIN ASSAYS
In several early studies a commercial serum-based immunoassay was used that measures the hepcidin precursor prohepcidin rather than the bioactive peptide (80). The relevance of these studies is questionable, because of the fact that prohepcidin concentrations correlate with neither urinary nor serum hepcidin concentrations, nor do they correlate with relevant physiological responses (71, 81–83). The first assays to measure bioactive hepcidin-25 were an immunodot assay (59), SELDI-TOF mass spectrometry (MS) (12, 15, 84). However, these assays could be used to measure hepcidin only in a semiquantitative manner.

SECOND-GENERATION (QUANTITATIVE) HEPCIDIN ASSAYS
In recent years, substantial progress has been made by the introduction of an internal standard to quantify hepcidin-25 in serum and urine by an updated TOF-MS assay (13, 30). In addition, Ganz et al. (29) developed a competitive (c)ELISA for human serum hepcidin. Other groups have also reported reliable hepcidin assays that can be divided in 3 main methodologies: (a) MS (85–95); (b) immunochemical assays, comprising cRIA (96, 97), cELISA (13, 29, 98, 99), and a 2-site ELISA (100); and (c) a ligand-binding assay (43) (Table 1). Of the currently available commercial immunochemical research kits for serum hepcidin, we found the RIA and enzyme-immunoassay kits of Bachem (purchased November 2009 and August 2010, respectively) to be suitable to differentiate between hepcidin concentrations in serum samples of controls and patients with various iron disorders, whereas the bioactive hepcidin kit of DRG Instruments, (purchased October 2009) gave similar concentrations for all samples and could not discriminate between iron metabolism disorders (unpublished results).

STRENGTHS AND LIMITATIONS OF QUANTITATIVE PLASMA AND URINE HEPCIDIN ASSAYS
MS assays require relatively expensive equipment, but they have the advantage of distinguishing between hepcidin-25, -22, and -20 (13). ELISA assays will measure total hepcidin concentrations, with (depending on the specificity of the antibody) different contributions from each of these 3 isoforms. However, this concern might be overcome by exploiting antibodies that are hepcidin-25 specific (100). At the same time it should be noted that the relevance of measuring hepcidin-25 instead of total hepcidin for clinical
decision-making has not been systematically investigated. Immunoassays have the potential for more widespread use in clinical laboratories. Furthermore, sample throughput is likely to be higher for ELISA than for MS assays. However, MS methods can also be automated (101, 102), and would have to be optimized for hepcidin because its amphiphatic character makes it readily stick to laboratory plastics, especially when relatively small sample volumes are used in large tubes.

Further comparison of the analytical characteristics of various published assays is incomplete, because not all studies have reported these characteristics in accordance with the STARD (STAndards for the Reporting of Diagnostic accuracy studies) procedures (103) or validated the reported assays according to recommendations of the International Conference of the Harmonization (ICH) of analytical procedures (104). Nonetheless, the scientific and medical community was informed on the status and agreement of the current hepcidin methods by a recent international send-out of samples for urine and serum hepcidin measurement in 2009, the so-called “Round Robin 1.” In this study the correlations between the participating methods were generally high and the between-sample and analytical variation of most methods were similar (105). However, absolute hepcidin concentrations differed widely between the assays. This latter variation is not surprising given the absence of a reference method (106) and a validated commutable calibrator or other material for assay harmonization. These differences in level assignment hinder the comparability of data collected by use of the various methods and preclude the definition of universal reference intervals and cutoff values for clinical decision making. Therefore, a second Round Robin for plasma hepcidin was initiated to explore word-wide hepcidin harmonization by exchanging synthetic materials for assay harmonization and native samples. Results are expected during 2011 (unpublished data).

Urine hepcidin measurements may be attractive, especially for use in research and measurements in children and patients in the underdeveloped world, because this matrix allows noninvasive sampling. Nevertheless, although a significant relationship was found between the hepcidin concentrations of urine–serum sample pairs from healthy controls and patients with disorders of iron metabolism (12, 29, 107), urine hepcidin concentrations may not always accurately reflect serum hepcidin concentrations. Interpretation of urinary hepcidin data is difficult because urine concentrations may also depend on glomerular filtration, tubular reabsorption, local production by tubular epithelial cells (25), and production by interstitial inflammatory cells (108). These issues make measurement of hepcidin somewhat disadvantageous in urine compared to serum and imply that for reliable interpretation of urine hepcidin concentrations as an alternative for serum concentrations, information on renal hepcidin production and handling is needed. Moreover, in a previous study we found a relatively high (pre)analytical variation for urine hepcidin measurements (107). Another potential drawback of urine hepcidin measurements is its sensitivity to oxidation (30) and the relatively high concentration of the smaller hepcidin isoforms, which cannot be distinguished from hepcidin-25 by most immunoassays. Altogether, these limitations should be kept in mind during the interpretation of urine hepcidin data and the decision to develop dedicated point-of-care devices for urine hepcidin measurements.

### Table 1. Currently available hepcidin methods.

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<td>Q-TOF LC-MS</td>
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<td>Altamura et al. (94)</td>
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<td>Ward et al. (95)</td>
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* UPLC, ultra-high pressure LC; MS/MS, tandem MS; Q, quadrupole; HBD, hepcidin-binding domain.
REFERENCE INTERVALS FOR SERUM HEPcidIN

Small and large studies of healthy controls have revealed considerable interindividual variation in hepcidin concentrations, resulting in wide reference intervals (12, 29, 96, 97, 107, 109). This situation suggests that reference intervals may have limitations when used for the interpretation of individual hepcidin concentrations. It appears that hepcidin values, like those of other hormones, should be interpreted in the context of other indices of iron metabolism. For instance, it is possible that “normal” concentrations of hepcidin in iron deficiency anemia (IDA) are inappropriately high, and that reference intervals may have limitations when used for the interpretation of individual hepcidin concentrations.

The wide reference intervals may be attributed at least partly to the increase of hepcidin concentrations in the course of the day (12, 29, 96, 97, 107). This apparent circadian rhythm of hepcidin concentrations may be regulated by transcription factors such as upstream stimulatory factor and c-Myc/Max through E-boxes (110). Genes that are regulated through E-boxes, including the clock genes period homolog 1 (Drosophila) (PER1), timeless homolog (Drosophila) (TIMELESS), and clock homolog (mouse) (CLOCK), tend to be under circadian rhythmic transcriptional control. However, circadian variation in hepcidin could be secondary as well, because it might be driven by the influence of iron intake during the day. As such, we recommend standardization of sampling time to minimize within-individual and (pre)analytical variability in clinical studies exploiting hepcidin measurements.

To date, differences in hepcidin concentrations between the sexes have not been consistently found (12, 29, 85, 96, 97, 107). We recently assessed serum hepcidin reference intervals stratified by age and sex stratified in a reference set selected from a large, well-phenotyped sample of the general population of the Dutch city of Nijmegen (109). We observed lower serum hepcidin concentrations in premenopausal women than in postmenopausal women (median 4.1 nmol/L vs 8.5 nmol/L) (109). Hepcidin concentrations in men were constant over age (median 7.8 nmol/L). In this study serum hepcidin was strongly associated with serum ferritin in men and women, confirming findings of smaller previous studies (29, 30, 107, 111, 112). Hepcidin concentrations have not been consistently compared between races and there are few studies in children (113–120). To the best of our knowledge there are 2 reported studies of hepcidin concentrations in pregnant women, both of which showed that hepcidin concentrations in anemic pregnant women is lower than in nonanemic pregnant women, but are not related to cord-blood or newborn hepcidin or other fetal iron parameters (114, 121).

As we discussed previously, basal hepcidin concentrations greatly depend on the applied measurement method (105). This situation implies that until harmonization is achieved, reference intervals as well as clinical decision limits for certain patient populations specific to the assay methods should be used. Harmonization of the methodologies, by using reliable calibrators or other materials for harmonization, will enable translational scientists as well as physicians in clinical practice around the world to collectively define criteria for the use of hepcidin assays in diagnosis, staging, monitoring, and assessing treatment indications of iron disorders.

Hepcidin in Human Iron Disorders

The development and validation of hepcidin assays paved the way for a large number of human studies that considerably increased our understanding of the physiology and pathophysiology of iron homeostasis and moreover demonstrated promising applications for hepcidin in diagnostic medicine (Table 2).

DISORDERS ASSOCIATED WITH HEPcidIN DEFICIENCY

Hepcidin in hereditary hemochromatosis (HH). HH comprises several genetic disorders of iron homeostasis characterized by body iron excess. This results in body iron overload, which can progress to organ injury, such as liver fibrosis and cirrhosis (122). Patients with most forms of HH have the inability to appropriately upregulate hepcidin synthesis in response to increased iron stores. This dysregulation is caused by defects in certain genes encoding for positive regulators of hepcidin (HFE, TfR2, HJV), or, rarely, the hepcidin gene [hepcidin antimicrobial peptide (HAMP)] itself [reviewed in (123, 124)]. The low hepcidin state relative to ferritin in these patients (125–127) leads to increased intestinal iron absorption and body iron overload. Hepcidin concentrations in untreated patients with rare juvenile forms of HH tend to be very low (127). Mutations in the gene encoding the cellular iron exporter ferroportin can also cause HH. However, there are 2 classes of ferroportin mutations with different phenotypic consequences: loss of hepcidin regulation and loss of ferroportin function (36, 128–130). The former defect leads to greater than normal ferroportin activity and thus is often described as a “gain of function” ferroportin mutation. As expected, patients with these mutations have a phenotype similar to classical HH. However, because the defect is downstream of hepcidin, these patients do not have low hepcidin concentrations. After treatment with phlebotomy that normalized iron stores (as assessed by serum ferritin), these patients have been found to have serum hepcidin...
concentrations or serum hepcidin/ferritin ratios within or above reference intervals (128, 131). The relatively high hepcidin concentrations in some patients might be a consequence of the transferrin saturation that remained increased despite phlebotomy. By contrast, mutational defects that result in the loss of ferroportin function cause cellular iron overload due to compromised iron export. Such patients thus have some phenotypic characteristics which vary from those of classical HH and have been reported to have high (urinary) hepcidin concentrations when untreated (127).

Iron-depleted HFE C282Y patients have been shown to have very low serum hepcidin concentrations, reflecting the combined effects of the HFE mutation and phlebotomy on hepcidin expression (125, 132). These patients demonstrated an exaggerated increase in serum iron concentrations upon oral iron loading (132). This observation draws further attention to the concern that lowering hepcidin concentrations by phlebotomy in HH patients may exacerbate the underlying pathophysiology, exacerbating the excess release of iron into the circulation, with an ensuing vicious circle that leads to the need for more frequent maintenance phlebotomies in HH patients (125, 126).

We foresee the following potential applications of hepcidin measurements in patients diagnosed with or suspected for HH: (a) screening for the presence of HH in patients with increased ferritin concentrations; (b) prioritization of genes to be investigated (very low hepcidin concentrations point to juvenile HH forms); (c) prediction of which C282Y-homozygous patients will be at risk for iron overload; and (d) monitoring of phlebotomy treatment (123, 133).

**Hepcidin in iron-loading anemias.** In congenital iron-loading anemias, such as β-thalassemia (major and intermedia) and congenital dyserythropoietic anemia I and II, but also in acquired forms such as myelodysplastic syndrome types RA (refractory anemia) and RARS (RA with ringed sideroblasts), the diseased erythron dysregulates iron homeostasis by inhibiting hepcidin synthesis, even in the presence of iron overload (134–140). The hepcidin/ferritin ratio is also found to be low in patients with these disorders (135, 137, 138, 140–142). The consequences of ineffective erythropoiesis on hepcidin expression thus appear to trump the consequences of excessive iron stores. In sickle cell disease, patients demonstrate the additional confounding factor of inflammation. Possi-

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**Table 2. Most promising applications of serum hepcidin in diagnostic medicine.**

<table>
<thead>
<tr>
<th>Disease condition</th>
<th>Expected hepcidin concentrations*</th>
<th>Potential added value of hepcidin diagnostics</th>
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</thead>
<tbody>
<tr>
<td>Classical hereditary hemochromatosis</td>
<td>Low, maybe compensated by iron overload over time</td>
<td>Screen for the presence of hemochromatosis</td>
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<tr>
<td></td>
<td></td>
<td>Predict which homozygotes will be at risk for iron overload</td>
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<td></td>
<td></td>
<td>Determine the phlebotomy interval</td>
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<tr>
<td></td>
<td></td>
<td>Prioritize genes to be investigated</td>
</tr>
<tr>
<td>Iron-loading anemias</td>
<td>Low, maybe compensated by transfusional iron overload over time</td>
<td>Identify the most severely affected patients</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Predict and monitor (parenchymal) iron overload</td>
</tr>
<tr>
<td>Acquired forms of iron overload</td>
<td>Miscellaneous</td>
<td>A marker of iron dysregulation</td>
</tr>
<tr>
<td>Iron-refractory iron deficiency anemia</td>
<td>Inappropriately high</td>
<td>Screen for primary defect in hepcidin regulation</td>
</tr>
<tr>
<td>Inflammation and infection</td>
<td>High</td>
<td>Differentiate ACD&lt;sup&gt;b&lt;/sup&gt; and IDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guide iron supplementation therapy</td>
</tr>
<tr>
<td>Chronic kidney diseases</td>
<td>High, decreases upon EPO treatment</td>
<td>Predict EPO response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guide treatment with EPO, intravenous iron</td>
</tr>
<tr>
<td>Acute kidney injury</td>
<td>Low (urine)</td>
<td>Diagnose acute kidney injury</td>
</tr>
<tr>
<td>Treatment with hepcidin antagonists and agonists</td>
<td>Depends on the disorder</td>
<td>Monitor and assess indications</td>
</tr>
</tbody>
</table>

*In serum, unless stated otherwise.
* ACD, anemia of chronic disease; IDA, iron deficiency anemia; EPO, erythropoietin.
Hepcidin in acquired forms of nonhemochromatotic iron overload. Several nonhereditary liver diseases such as excessive alcohol consumption, nonalcoholic fatty liver disease and nonalcoholic steatohepatitis are associated with mild hepatic iron overload. In these so-called nonhemochromatotic iron overload diseases, hepcidin concentrations are increased, but relatively low for the level of iron overload, suggesting ineffective liver iron sensing. In these conditions, dysregulation of hepcidin synthesis as a consequence of reactive oxygen species, endoplasmic reticulum stress, and cytokine tumor necrosis factor-α–mediated pathways may contribute to relatively decreased hepcidin synthesis and iron accumulation (59, 67–70, 144). Measurement of serum hepcidin may prove to be useful as a marker of iron dysregulation in these forms of iron overload.

Hepcidin in IDA. In IDA patients, and also in patients with low ferritin concentrations without anemia, secondary to blood losses or insufficient dietary intake, hepcidin concentrations have been found to be very low, often below the lower limit of detection of the employed assays (13, 29).

Effect of iron administration on hepcidin concentrations. Several studies have addressed the relationship between iron absorption and hepcidin concentrations. In healthy human volunteers hepcidin in urine and serum increased upon oral iron dosing, although nonresponders were reported (29, 59, 112, 145–147). Some studies report a weak, but significant, negative correlation between serum hepcidin concentrations and iron absorption (112, 146, 147). These results suggest that quantification of serum hepcidin concentrations could be a predictor for the therapeutic effect of oral iron administration. Moreover, the response of the hepcidin to oral iron might prove to be a useful test to evaluate iron absorption in iron deficiency.

DISORDERS ASSOCIATED WITH HEPcidIN EXCESS

Hepcidin in iron-refractory IDA. Most patients with iron-refractory IDA are resistant to dietary iron supplementation, owing to a defect in the TMPRSS6 gene encoding matriptase-2. Repeated intravenous iron infusions, on the other hand, have been reported to (partially) increase hemoglobin and mean cell volume (119, 120, 148–151). Loss of matriptase-2 activity results in an inability to reduce hepcidin synthesis during iron deficiency (49). Accordingly, patients have increased innate hepcidin concentrations resulting in extremely low transferrin saturation percentages and ferritin concentrations in the lower range of the reference interval (118, 119, 120, 148–151). Therefore, assessment of hepcidin concentrations can serve to exclude or raise the suspicion for matriptase-2 deficiency in unexplained IDA and thus help to avoid the unnecessary burden associated with multiple diagnostic testing and delayed or ineffective treatment.

Hepcidin in infectious and inflammatory diseases. Infections are associated with marked changes in iron homeostasis (152). Malaria-associated anemia has been found to be associated with low serum iron and increased hepcidin concentrations, causing iron withholding in the RE system that ultimately leads to iron-restricted erythropoiesis (115, 118, 153–156). Possibly, these increased hepcidin concentrations might be advantageous in the protection against extracellular growing microorganisms by decreasing the extracellular supply of iron. On the other hand, increased hepcidin concentrations may lead to iron deficiency and blunt the efficacy of iron fortification programs by decreasing iron absorption (157). Conversely, the increased hepcidin concentrations seen with inflammation might increase the virulence of bacteria that depend on macrophage iron for their proliferation within these cells (e.g., Salmonella and Mycobacteria spp.). Such pathogens are common in malaria-endemic regions. Perhaps, in such areas, untargeted iron supplementation among iron-replete children might result in severe adverse events by increasing the proliferation of latent pathogens (158).

Hepcidin has been demonstrated to be important in the pathogenesis of other infectious agents as well. In patients with hepatitis C virus, increased transferrin saturation and increased concentrations of serum ferritin and hepatic iron have been observed. Consistent with this iron loading, low hepcidin concentrations have been found in these patients, along with other changes in the expression of iron-transport genes [reviewed in (159)]. In a pediatric study, no relationship was found between Helicobacter pylori infection and hepcidin and between cytokine and hepcidin concentrations, suggesting that H. pylori–induced inflammation does not influence iron status through increased hepcidin production in childhood (118).

In ACD, IL-6 and other cytokines induce hepcidin concentrations, resulting in iron sequestration in macrophages and leading to hypoferremia. Subsequently, iron availability for the erythron becomes limiting.
Hepcidin as a Biomarker in Iron Disorders

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and contributes to the development of ACD (59, 75, 81, 160, 161). Elderly people often suffer from anemia. In a recent study among elderly individuals (≥65 years old), the presence of anemia could not be attributed to increased hepcidin concentrations, because urinary hepcidin concentrations were significantly lower among participants with inflammation anemia and iron deficiency compared with nonanemic controls. This finding raises the possibility that in this population hepcidin-independent mechanisms can cause hypoferremia and anemia of inflammation, and/or that even otherwise “normal” hepcidin concentrations are sufficient to sustain anemia once it is initiated (162).

At present, there is no single laboratory test that can definitively distinguish ACD from IDA (160, 163). Therefore, differentiation between these conditions is often achieved by combining various biochemical markers of iron metabolism. Studies to assess the role of hepcidin in ACD are complicated by the lack of a gold standard methodology. As a result the outcomes of studies to assess the diagnostic value of hepcidin largely have depended on the definition of surrogate gold standards. Some studies showed that hepcidin is an appropriate marker in the differentiation of ACD and IDA/ACD but not of IDA and IDA/ACD (75), whereas others have reported that hepcidin allowed distinguishing IDA from IDA/ACD but not ACD from IDA/ACD (13, 164). Results of another study have suggested that hepcidin concentrations can serve as an appropriate marker to differentiate between all 3 groups (165).

Hepcidin measurements appear to have potential utility in diagnostic algorithms to distinguish IDA from ACD and the combined presence of IDA/ACD. Further studies in which hepcidin response to iron supplementation is used as a proxy for the gold standard of iron status would be informative. Hepcidin measurement also may be useful in anticipating consequences of iron supplementation in infectious and inflammatory diseases.

Hepcidin in renal diseases. The introduction of ESAs, such as EPO, has allowed effective treatment of anemia in patients with CKD. However, the optimal target concentration of hemoglobin is debated, and many patients become ESA resistant. Soon after its discovery and during the years thereafter, hepcidin was recognized as potentially relevant in CKD because it may be responsible for the often-observed imbalance between iron homeostasis and ESA resistance (50, 166). As such hepcidin was described as a promising companion diagnostic marker to predict ESA responsiveness, and to guide treatment with ESA and intravenous iron. In addition, hepcidin’s potential to become a target of treatment was quickly acknowledged. These promises brought about a large series of studies that examined hepcidin concentrations in patients with CKD. In general, these studies have reported increased hepcidin concentrations in CKD patients compared with healthy controls (15, 16, 29, 31, 32, 88, 89, 96, 167–169). Because these patients tend to be anemic, even a mild hepcidin excess can be considered to be inappropriately high for the level of anemia. Virtually all studies on hepcidin in CKD patients revealed a strong positive relationship between hepcidin and ferritin concentrations. Some of these studies also demonstrated correlations between hepcidin and iron or transferrin saturation (169, 170). Hepcidin in CKD also has been associated with iron-restricted erythropoiesis, as reflected by the relation of hepcidin with lower hemoglobin and/or reticulocyte counts (31, 169, 171). Furthermore, CRP and IL-6 were shown to be less relevant predictors in the setting of renal insufficiency (16, 170, 172–175). Intravenous iron administration was found not to influence hepcidin concentrations in hemodialysis (HD) patients that were withheld from iron and ESA therapy for 2 weeks (169), whereas it increased hepcidin in iron-naïve CKD patients (31, 96). In HD patients on ESA, hepcidin did not predict which patients increase their hemoglobin after iron loading (17). As mentioned earlier, several investigators have reported a decrease in hepcidin concentrations after EPO administration (31, 50, 169, 170). These findings again suggest that the erythroid demand for iron might be a more powerful regulator of hepcidin expression than inflammatory- or iron-induced hepcidin formation. To allow proper interpretation of the effect of the erythropoietic drive on hepcidin production, EPO and hepcidin concentrations should be assessed simultaneously in selected patients with anemia of CKD.

Interestingly, hepcidin also was evaluated as a predictor of ESA response in several relatively small studies in humans. One study revealed that hepcidin concentrations of EPO responders did not differ from those of hyporesponders (176), whereas cross-sectional studies among HD patients and a prospective study among patients with combined CKD and chronic heart failure revealed that nonresponders had low hepcidin concentrations (32, 170, 177). Possibly hepcidin should be regarded as a marker of response rather than resistance to ESA treatment in these patients.

Although hepcidin is a promising companion diagnostic for ESA therapy, patients with renal insufficiency are a rather complex population in which consistent results have been difficult to obtain (175). Clinical stability, time of sampling in relation to iron and ESA therapy, and iron and EPO dosage and dialysis
Hepcidin in obesity-related diseases. Adipose tissue is an active endocrine organ that releases several cytokines and adipokines, which can contribute to the development of a low-grade systemic inflammation. Furthermore, adipose tissue also can produce hepcidin (21). The low-grade inflammation in obese and dysmetabolic iron overload syndrome patients, (characterized by the association of increased body iron stores and metabolic features), is associated with increased hepcidin concentrations, leading to poor iron absorption and causing ACD (116, 117, 180, 181). Serum hepcidin concentrations decreased, and an increase in iron status and intestinal absorption was observed in individuals with weight loss (182, 183). Thus, in chronic mild inflammatory conditions, such as obesity or the metabolic syndrome, even a mild hepcidin excess may be sufficient to alter the balance between iron loss and iron uptake toward iron deficiency.

Hepcidin in heart diseases. Anemia of chronic heart failure has been found to be associated with low hepcidin concentrations, making (low-grade) inflammation less likely as the sole underlying mechanism (184, 185). A study on acute cardiac ischemia showed a transient increase in serum hepcidin-20 concentrations for the majority of patients studied, whereas hepcidin-25 remained increased 7 days after the incident. The authors concluded that hepcidin-20, rather than hepcidin-25, may be helpful for the diagnosis of acute myocardial infarction (14).

Several reports suggest the potential use of hepcidin concentrations in determining cardiovascular risk. It has been hypothesized that increased hepcidin concentrations may enhance cardiovascular risk by increasing intracellular macrophage iron concentrations and by increasing their atherogenic potential (186-189). Furthermore, recent studies showed increased hepcidin production in patients with (low-grade) inflammation, obesity, and nonalcoholic fatty liver disease, which are traditionally associated with cardiovascular disease (21, 144, 190, 191). Additional data were reported that suggested an atherogenic function of hepcidin in a study that revealed that ex vivo hepcidin-25 treatment of differentiating monocytes from patients with the metabolic syndrome induced monocyte chemoattractant protein-1 (192). Moreover, hepcidin concentrations in these patients were associated with increased concentrations of monocyte chemoattractant protein-1 and vascular damage. These observations raise the possibility that hepcidin may prove to be a novel determinant for cardiovascular disease risk.

Hepcidin in cancer. Patients with cancer often suffer from anemia, generally in association with other markers of inflammation. Studies in patients with multiple myeloma suggest that hepcidin is upregulated in these patients by both IL-6-dependent and IL-6-independent mechanisms that may play a role in the anemia often observed in these patients. The IL-6-independent pathway may be attributed to increased BMP-2 concentrations, which have been suggested to act synergistically with IL-6 to induce hepatocyte hepcidin synthesis (193, 194).

For Hodgkin lymphoma we recently demonstrated that increased IL-6 production is associated with induction of hepcidin, which contributes to the iron-restricted anemia of chronic disease often observed at Hodgkin lymphoma diagnosis. However, in this study increased hepcidin concentrations appeared insufficient for the development of anemia (195), suggesting that compensation by increased erythropoiesis through increased EPO production may explain the lack of anemia in cancer despite increased hepcidin concentrations (196).

In a study among anemic cancer patients receiving ESA therapy, those with relatively low hepcidin concentrations at baseline showed a better response (197). Surprisingly, these results are not fully consistent with those found for patients with CKD (see above), but suggest that baseline hepcidin concentrations may play a potential role as a predictive marker to identify patients who either are going to respond adequately to EPO therapy or need to be excluded from anemia correction with ESA owing to a low chance of responding.

Although the number of studies on the role of hepcidin in the anemia of cancer is limited, these studies have contributed to our insights regarding the mechanisms involved. Future studies are needed to assess the position of hepcidin in clinical decision making in these patients.
Hepcidin in sports medicine. The prevalence of iron deficiency is high among athletes. A number of exercise-generated mechanisms may contribute to this low iron status of athletes, including hemolysis, hematuria, sweating, and gastrointestinal bleeding (198). Recently, several (199–201) but not all (202) studies showed increased urinary hepcidin concentrations after exercise. These findings of increased hepcidin concentrations led Peeling et al. to propose that exercise-induced increases in hemolysis and hepcidin expression may have 2 effects. First, any postexercise free iron uptake by the RE macrophages may be trapped within the scavenger cell owing to the action of hepcidin on the ferroportin transporters, thereby affecting the recycling of iron back into the circulation. Second, increased hepcidin activity postexercise may reduce the absorption of iron from the diet (203). Hepcidin might thus be a mediator of the high incidence of iron deficiency among athletes.

Hepcidin-Modulating Agents

Hepcidin-targeted therapies may improve treatment options for patients suffering from iron disorders. Although no specific hepcidin therapies are available, several compounds are under development as hepcidin agonists or antagonists [reviewed in (204–206)]. Hepcidin agonists could be useful to prevent iron overload attributed to hepcidin deficiency, such as HH, and especially β-thalassemias and other iron-loading anemias, and possibly some acquired forms of non-hemochromatotic iron-overload diseases. Hepcidin antagonists, on the other hand, might be expected to benefit patients with diseases of hepcidin excess manifested as iron-restricted anemia and systemic iron deficiency, such as iron-refractory IDA, ACD (rheumatic diseases, inflammatory bowel diseases, autoimmune diseases), CKD, multiple myeloma and other cancers, obesity-related iron deficiency, and cardiovascular disease.

Hepcidin agonists

Preclinical studies and some clinical studies have provided proof-of-concept for synthetic hepcidin–25, small hepcidin peptides, BMP agonists, and HIF stabilizers to prevent iron overload attributed to hepcidin deficiency.

Small hepcidin peptides have been shown to act as agonists in mice in vivo on the basis of their aminoterminal domain (206). Gardenghi et al. reported that transgenic hepcidin overexpression in β-thalassemia intermedia mice decreased iron overload, and even ameliorated ineffective erythropoiesis (207).

Hepcidin concentrations can also be induced by targeting the BMP pathway (Fig. 4). BMP-2 administration in mice was found to increase liver hepcidin expression and reduce serum iron concentrations in vivo (208). Supraphysiological doses of exogenous BMP-6 for 10 days also were reported to improve hepcidin deficiency, reduce serum iron, and redistribute tissue iron to appropriate storage sites (RE macrophages, spleen) in a mouse model for Hfe hemochromatosis (209). HIF antagonists are used in cancer to inhibit tumor angiogenesis (210). Blocking HIF could also be of useful for increasing hepcidin concentrations in iron-overload diseases.

Hepcidin antagonists

Preclinical and clinical studies have also shown that hepcidin antagonists such as hepcidin antibodies, BMP antagonists, cytokine receptor antibodies, and HIF stabilizers can decrease hepcidin expression and reverse iron abnormalities (208, 211–218).

In a proof-of-principle study, Sasu et al. neutralized hepcidin by a monoclonal antibody in the Brucella abortus mouse model of anemia of inflammation and found it to restore responsiveness to EPO (211). The results of this study suggest that administration of antihepcidin therapies alone or in combination with erythropoiesis-stimulating agents may improve patients’ erythropoietic response and allow the use of lower EPO doses to avoid the potential detrimental effects of high EPO concentrations. Notably, monitoring of the bioactive serum hepcidin during treatment by these hepcidin antagonists appears to be complicated by the interference of the compounds in the assays (219).

Several components can interfere with the BMP/SMAD pathway. Administration of dorsomorphin, a small inhibitor of BMP signaling, prevented hepcidin induction by iron in mice (212). GDF15 and TWSG1 are both BMP antagonists produced by erythroblasts and putative components of the erythroid regulator that can inhibit hepcidin expression in vitro. Although the physiological relevance of GDF15 and TWSG1 remains to be defined, these proteins might have therapeutic potential. Soluble HJV, which also acts as an antagonist of BMP signaling, decreases hepcidin baseline expression in mice, mobilizes RE-system (spleenic) iron stores, increases liver iron content and concurrently increases serum concentrations (208). Also, heparin and heparin derivatives have recently been shown to inhibit hepcidin expression in vitro and vivo by interfering with BMP signaling (218).

Anti–IL-6–receptor antibody (tocilizumab) was shown to suppress IL-6–induced hepcidin production in Castlemann disease (a rare disorders with systematic inflammation and anemia), and it improved anemia in arthritic monkeys (214–217). Inhibitors of the key initiators of the cellular hypoxic response, such as prolyl...
hydroxylases, prevent inactivation and degradation of HIF and as such may be effective hepcidin suppressors and restore natural iron regulation in anemia (220, 221).

There is an increasing demand for preclinical tools to assess the efficacy of hepcidin-related therapies. Therefore, the diagnostic toolbox for hepcidin peptide measurements in animal models should be improved and extended (75, 219, 222). Moreover, well-designed large clinical studies addressing safety and long-term efficacy are needed to clarify the risks and benefits of hepcidin-targeted treatments.

Concluding Remarks

Since the discovery of hepcidin 10 years ago, multiple studies have contributed insights into the regulation of hepcidin and its functional properties. The first reliable assays to quantify hepcidin in human body fluids have recently been developed, and proof-of-principle studies in human iron disorders highlight hepcidin as a promising novel tool in diagnostic medicine, as outlined in Table 2. However, the use of different assay technologies to assess hepcidin concentrations in patients with disorders of iron metabolism makes it difficult to interpret the results of the many reported studies. For example, the accumulation of hepcidin-22 and -20 isoforms may raise questions regarding the interpretation that measured hepcidin concentrations reflected bioactive hepcidin-25 in studies in which certain immunoassays were used. Moreover, there is a need for harmonization of the various assays to enable the establishment of world-wide reference intervals and clinical decision limits. Large and well-designed studies exploiting harmonized assays are required to more firmly establish the position of hepcidin in diagnostic medicine. Furthermore, as the field moves toward the use of hepcidin-related therapies, it should be realized that the outcome of assays that are used to monitor the fate of hepcidin during antihepcidin therapy may be affected by compounds that interfere with hepcidin. In cases in which interference has occurred, assays must be revalidated for this specific application before definite conclusions on the efficacy of the therapy can be drawn. In conclusion, hepcidin is a promising diagnostic tool but efforts must be undertaken to assess the relevance of specifically measuring hepcidin-25, to harmonize assay outcomes throughout the world, to define clinical decision limits, and to make assays available to clinical laboratories before hepcidin assays can be fully included in clinical practice.

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