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Hepcidin: from discovery to differential diagnosis
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ABSTRACT

Although iron is essential for living organisms to survive, its reactive properties require strict regulation in order to prevent toxic effects. Hepcidin, a liver produced peptide hormone, is thought to be the central regulator of body iron metabolism. Its production is mainly controlled by the erythropoietic activity of the bone-marrow, the amount of circulating and stored body iron, and inflammation. Recent reports, however, provide new hypotheses on how hepcidin might exert its regulatory function. Although hepcidin was first discovered in human urine and serum, most of our understanding of hepcidin regulation and action comes from in vitro and mice studies that often use hepcidin mRNA expression as a read out. The difficulties in carrying out studies in humans have mostly been due to the lack of suitable hepcidin assay. The recent development of assays to measure hepcidin in serum and urine has offered new opportunities to study hepcidin regulation in humans. However, for the moment, only a small number of laboratories are able to perform these assays. The aim of this review is to discuss insights into hepcidin regulation obtained from recent clinical studies in the light of findings from in vitro and mice studies. Ongoing studies in humans should provide us with more information on the etiology of iron metabolism disorders in order to create new therapeutic strategies and improve differential diagnosis protocols for these diseases.

Key words: hepcidin, iron.

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Introduction

Body iron metabolism is based on a highly efficient system of iron conservation and recycling by which only a part of the daily need is replaced by duodenal absorption (Figure 1A). Maintenance of body iron homeostasis requires mechanisms to control uptake and mobilization from stores to meet erythropoietic needs and to scavenge previously used iron. Therefore, the communication between cells that consume iron and cells that acquire and store iron must be strictly regulated.2 Duodenal enterocytes absorb iron from the diet, where it is mostly present as ferric Fe3+ or as heme (Figure 1B). After reduction on the apical surface of the enterocytes by duodenal cytochrome-b (Dcytb), iron enters the cell by divalent metal transporter-1 (DMT-1). Heme is reported to be absorbed by a recently identified receptor heme-carrier protein-1 (HCP-1)3 and released from iron by heme oxygenase-1 (HO-1). Once in the cell, iron is stored by ferritin and released into the plasma by the cellular iron exporter ferroportin when there is high body demand. After oxidation by ferroxidase hephestin (intestinal cells) or ceruloplasmin (nonintestinal cells), iron is loaded onto transferrin for transport in the plasma where it is picked up by TfR1 on the cell surface of cells in need of iron. Besides dietary uptake, iron is recycled from senescent erythrocytes by macrophages (Figure 1C). Also here, HO-1 plays an important role in extracting iron from heme in the cytosol while involvement of DMT-1 is suspected but not proven.4 A recent review on cellular iron uptake, storage and export is given by Dunn et al.5

The discovery of hepcidin

Although hepcidin was first discovered in human urine and serum, most data on hepcidin expression, regulation, structure and function were obtained by in vitro approaches and studies in mice. Hepcidin was initially isolated from plasma ultrafiltrate6 and named liver-expressed antimicrobial peptide (LEAP-1). Around the same time, it was isolated from human urine and named hepcidin after its hepatic origin and bactericidal effect in vitro.7 The development of severe iron overload8 by knocking out the gene in mice suggested that hepcidin is involved in iron metabolism, whereas this key role in regulation was underlined

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by the discovery of hepcidin mutations in patients. The newly discovered peptide was found to be regulated by inflammation, iron stores, hypoxia and anemia.

Structure of hepcidin
The human hepcidin gene (HAMP; OMIM 606464), located on chromosome 19q13.1, encodes a precursor protein of 84 amino acids (aa) During its export from the cytoplasm, this full-length pre-prohepcidin undergoes enzymatic cleavage, resulting in the export of a 64 aa prohepcidin peptide into the ER lumen. Next, the 39 aa proregion peptide is probably posttranslationally removed by a furin-like proprotein convertase resulting in mature bioactive hepcidin-25 (25 aa form). In human urine, Park et al. also identified hepcidin-22 and hepcidin-20, which are N-terminally truncated iso-forms of hepcidin-25. Recently we confirmed that in addition to hepcidin-25, the 20 aa iso-form is detectable in both human urine and serum, while the 22 aa iso-form can only be detected in urine. These results support the hypothesis that the 22 aa peptide is merely an urinary degradation product of hepcidin-25.

Structural analysis of human synthetic hepcidin by NMR spectroscopy revealed that this 8 cysteine-containing peptide forms a hairpin-shaped molecule with a distorted β-sheet which is stabilized by four disulfide bridges between the two anti-parallel strands. One of the disulfide bridges is located in the vicinity of the hairpin loop which points to a possible crucial domain in the activity of the molecule (Figure 2). The high cysteine content of the molecule is highly conserved among other species. Structure-function in vivo (mice) and in vitro studies on synthetic hepcidin have shown that the iron regulating bioactivity is almost exclusively due to the 25 aa peptide, suggesting that the five N-terminal amino acids are essential for this activity.

In vitro experiments have shown that especially human hepcidin-20 exerts anti-bacterial and anti-fungal activity in a concentration range 10-fold higher than that measured in healthy individuals. Therefore, it is not clear whether in vivo hepcidin levels can reach values in which it can be anti-microbial, or whether this function is of biologic importance or only rudimentary in its evolutionary origin. A recent report on inductively coupled plasma-mass spectrometry (ICP-MS) on human hepcidin extracted from urine samples showed co-purification of at least one iron molecule with hepcidin. Modeling of a best-fit 3D structure of hepcidin with iron demonstrated significant differences from the previously reported synthetic hepcidin model. These new findings suggest a conformational polymorphism for hepcidin as a regulatory mechanism for iron uptake as part of its role as regulator of iron homeostasis.

Mechanism of hepcidin activity
Recently, hepcidin was reported to bind to the transmembrane iron exporter ferroportin which is present on macrophages and the basolateral site of enterocytes (Figure 1B and C), but also in hepatocytes. It has been demonstrated in vitro that hepcidin induces the internalization and degradation of ferroportin. It is not yet known whether structural properties such as amino terminal Cu and Ni binding motifs (ATCUN) motifs in the hepcidin-25 molecule play a role in this process (Figure 2). For cellular iron export, the cell depends on ferroportin, as originally demonstrated in zebra fish. By diminishing the effective number of iron exporters on the membrane of enterocytes or macrophages, hepcidin suppresses iron uptake and...
release respectively. This is in line with the phenotype of ferroportin disease where iron accumulation is observed mainly in macrophages often combined with anemia.  

Besides this systemic liver hepcidin controlled reduction of iron uptake and release, there is also evidence for local production of hepcidin by macrophages, fat cells and cardiomyocytes. These findings suggest hepcidin is involved in different regulatory mechanisms to control iron imbalance.

**Hepcidin at the nexus of various regulatory pathways**

At present, four putative upstream regulatory pathways are generally thought to control liver hepcidin production: (i) iron store-related regulation (ii) erythropoietic activity driven regulation (iii) inflammation related regulation, and (iv) a mandatory signaling pathway. All are found to interact with liver cells to initiate the production of sufficient hepcidin for correct maintenance of iron homeostasis. A model of pathways involved in hepcidin regulation that builds upon recent findings mainly from mice studies and in vitro cell culture studies is shown in Figure 3. Three relevant sites involved in hepcidin regulation are presented: kidney, bone marrow and liver cells. Notably, the iron efflux regulation in macrophages by hepcidin and skeletal muscles expressing high levels of hemojuvelin are not shown. A detailed discussion of each of these 4 pathways and the transcriptional regulation of hepcidin can be found in Online Supplemental text file 1.

**Hepcidin kinetics**

After hepcidin is secreted into the circulation by the hepatocytes it eventually exits the body in the urine. Its effect on the decrease in serum iron levels in mice appeared to take place within 4 hours in a dose-dependent way that was sustained for more than 48 hours. Indirect hepcidin inductions by IL-6 or LPS in humans displayed the same fast response in urinary hepcidin excretion, thereby acting like an acute phase protein with a peak value after 6 hours, followed by a steady decrease. Oral iron administration for 3 days in healthy human volunteers showed a significant increase in urinary hepcidin after 24 hours which disappeared over the following days despite the iron intake. The peak urine value suggests a fast clearance of hepcidin from the circulation, with a paradoxical sustained inhibitory effect on iron uptake, shown by the iron parameters which remained unchanged over the following days.

The fast appearance of hepcidin in urine demonstrates the exceptional characteristics of this peptide. So far, tubular dysfunction, or even reported tubular expression of hepcidin as a causative factor in hepcidinuria, have not yet been studied.

**Hepcidin: from mice to men**

Although highly homologous hepcidin genes have also been identified in many vertebrates, including rodents like mice and rats, several species of fish, dogs and pigs, it should be noted that in mice two paralogous genes have been found from which only Hepc1 appears to be involved in iron metabolism. As previously described, mice have provided a useful animal model to identify and trace the pathways in iron metabolism, but these differences at the genetic level of hepcidin mean that care must be taken when considering results on hepcidin obtained in mice in studies on humans. Similarly, although in vitro systems allow a broad spectrum of intervention, any similarity to the human in vivo situation is limited. Therefore, measurement of circulating levels of hepcidin in man is important to increase our knowledge on the role of hepcidin in different pathological conditions.
Table 1, panel A). More specifically, these findings might add to the definition of hepcidin as a differential diagnostic tool and therapeutic target in human disease.

Assessment of urinary and serum hepcidin in humans

Until recently, few investigative tools were available to detect hepcidin in human studies. Hepcidin mRNA expression is mostly preferred in animal and cell culture studies, but is obviously used very sporadically in human studies because of the need of invasive sampling. Immunochemical methods based on the use of specific anti-hepcidin antibodies, such as in immuno-histochemical tissue staining, SDS-PAGE and Western Blot\textsuperscript{41,42} are problematic mainly because of the limited availability of suitable antibodies. This can be attributed to the small size of hepcidin, the compact and complex structure of the molecule, and the highly conserved sequence among species, complicating the elicitation of an immune response in host species. We will briefly describe and discuss the assays currently available to measure (pro)hepcidin in serum and/or urine.

Antibody-based hepcidin assay

To our knowledge, only one antibody-based dotblot assay has successfully been used so far to semi-quantify hepcidin in urine.\textsuperscript{38} With the use of cation exchange chromatography, peptides are extracted from urine and resuspended and immobilized on a vinyl membrane. Hepcidin is quantified by Chemiluminescence detection using rabbit anti-human hepcidin primary antibodies,\textsuperscript{41} and goat anti-rabbit horseradish peroxidase as a secondary antibody. Hepcidin quantity in each sample is normalized using urinary creatinine concentrations. Given the lack of a control for hepcidin losses in the pre-analytical phase of the analysis, such as in the protein extraction and resuspension steps, this assay could be considered semi-quantitative. The limited availability of mostly non-commer-
ment of its precursor pro-hepcidin has been reported with the use of a commercially available ELISA kit that uses antibodies directed against the pro-peptide region of hepcidin. However, the diagnostic use of this assay is controversial because of the lack of clear correlations with hepcidin and other iron related parameters. Significant concentration differences only have been reported in ferroportin disease or in combination with end-stage renal disease (ESRD).

Mass spectrometry-based hepcidin assay

Recently we described a surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)-based assay that detects the three known isoforms of hepcidin. More recently improvements have been made in this technique suitable for measuring hepcidin-25 in serum. Importantly, the procedure is quick to carry out but difficult to robotize due to the on-spot procedure. The latest improvement in the application includes both a procedure to prevent hepcidin losses during sample preparation and the introduction of a hepcidin analogue as internal standard, which allows sensitive quantification of hepcidin in both serum and urine samples (D. Swinkels and C. Lasaakkers, unpublished results).

The potency of MS-based hepcidin analysis has been underlined in other recent reports. Tomosugi and co-workers reported serum hepcidin measurements with the use of SELDI-TOF MS, but with the use of diluted serum samples and a bioprocessor. Compared to the direct on-spot method described above, this application allows the use of robotics. It uses synthetic human hepcidin-25 to construct an external standard curve, but since it doesn’t use an internal standard it should be regarded as semi-quantitative.

Another report on serum hepcidin analysis uses liquid chromatography tandem mass spectrometry (LC-MS/MS) with a non-hepcidin related internal standard. Both LC-MS/MS and SELDI-TOF MS still need highly specialized equipment, and therefore do not readily improve accessibility.

Comparative studies, the use of uniform internal standards, and accuracy adjustment of all techniques available to date might increase the comparability of results in the future. For the moment, mass spectrometry-based hepcidin measurements in serum and urine seem to be an attractive option to semi-quantify serum and urine hepcidin levels in clinical studies for research purposes in a small number of laboratories around the world.

A constant improvement in analytical techniques is essential to accurately assess levels of hepcidin and its isoforms in body fluids of different species, cell media and cell contents.

Insights from hepcidin measurements in humans

The development and implementation of urinary and serum hepcidin assays led to several clinical studies that increased the existing knowledge on hepcidin levels in physiologic and pathophysiologic states. These studies will now be discussed in the light of previous related in vitro approaches and analogous studies using mice as model organisms. An overview is presented in the Online Supplemental Table.

Pre-analytical factors and diurnal variation

First of all, evaluation of our MS-based hepcidin measurements in human serum and urine samples revealed a substantial influence of pre-analytical factors, especially on urinary hepcidin, and a large diurnal variation in serum hepcidin levels. Murphy et al. also found a large diurnal variation. In mice, background influences like strain and gender have been shown to substantially modulate liver hepcidin mRNAs. In a small set of samples, Murphy et al. found no major differences between serum concentrations from normal males and females, which agrees with our findings in a different control group for both serum and urine values.

Hepcidin regulation

The involvement of hepcidin in the induction of hypoferreraemia by inflammation was translated from mice to human studies after the introduction of the urinary dot-blot and the SELDI-TOF MS assays, which also demonstrated the existence of the highly responsive LPS-IL-6-hepcidin axis as a link between innate immunity and iron metabolism.

Erythropoietic activity appeared to be a strong regulator of hepcidin levels, i.e. erythropoietin that stimulates erythropoietic activity has been shown to down-regulate liver hepcidin mRNA-expression in mice. However, in the absence of erythropoietic activity, hepcidin expression is no longer suppressed. The strong inverse association between erythropoietic drive and hepcidin production was also observed in several patients with congenital chronic anemias, which are characterized by low urinary hepcidin levels.

Hepcidin in hereditary hemochromatosis

Both SELDI-TOF MS and the dotblot method were used to measure hepcidin levels in juvenile cases of hemochromatosis, and were found to be extremely low. Less severely decreased, but still clearly low urinary hepcidin levels, were found in patients with TR2 mutations using the urine dotblot method. Similarly, decreased mRNA levels were seen in liver biopsies of HFE-KO or deficient mice and humans, whereas levels ranging to almost normal values in serum and urine were found in HFE-hemochromatosis patients. Although the mechanism behind this observed variability in hepcidin values especially in classic hemochromatosis is still not completely clear, the presence of iron overload at presentation and of the increased erythropoiesis with phlebotomy treatment are likely to contribute (B. van Dijk and D. Swinkels, unpublished observations).

In ferroportin disease, hepcidin concentrations appear...
to vary with the sequence variations of the gene and the way they influence the activity of the ferroportin protein.\textsuperscript{16,20} Patients with 162delVal and N144H alterations in ferroportin were reported to have high hepcidin levels with a loss in function of ferroportin,\textsuperscript{13,63} whereas in cases of other variants with a gain of function the hepcidin levels are normal but relatively too low for the degree of iron loading in these patients.\textsuperscript{12,21}

**Hepcidin in secondary iron overload**

The dotblot and SELDI-TOF methods were used to observe low/normal hepcidin levels for the degree of iron load in thalassemic patients,\textsuperscript{17,53-56} which agree with levels of expression found in mice\textsuperscript{64-66} and are thought to be the cause of increased iron uptake. Most of these patients are transfusion dependent, thereby aggravating the iron burden.\textsuperscript{48} The lower hepcidin levels mostly found in thalassemia intermedia compared with thalassemia major can be attributed to the higher degree of erythropoiesis expansion and lower degree of iron burden of the intermedia form.\textsuperscript{56}

**Hepcidin and renal anemia**

SELDI-TOF MS measurement of serum hepcidin in end-stage renal disease patients showed accumulated hepcidin levels that could be partly reduced by hemodialysis.\textsuperscript{49} This accumulation suggests hepcidin contributes to the pathogenesis of renal anemia. Much of the etiology behind this anemia is still unclear, but it is thought to be caused by reduced EPO production and is, therefore, mostly treated by administration of human recombinant erythropoietin (EPO) in combination with oral iron. Other factors, such as hyperparathyroidism, aluminum toxicity, systemic inflammation, and impaired iron metabolism, seem to be of minor importance.\textsuperscript{49}

**Differential diagnosis and therapeutic implications**

The outcomes of the studies in humans described above have implications for the use of hepcidin as a diagnostic and therapeutic tool. These are based on small series, sometimes even on only a few cases. This shows that large scale clinical validation is still needed to prove the power of hepcidin in differential diagnosis. First, the circadian rhythm observed for serum might require sampling protocols for hepcidin analysis that include standardization of time of blood withdrawal, similar to that for the assessment of serum iron levels or transferrin saturation.

Second, recognition of iron deficiency anemia (IDA) in the context of anemia of chronic disease (ACD) is currently performed with routine biochemical parameters, such as transferrin saturation, ferritin, CRP and, less often, soluble TfR (sTfR), zinc protoporphyrin and new erythrocyte indices. Each have their own disadvantages.\textsuperscript{16,20} In contrast to increased levels of hepcidin in ACD, both in vitro iron deficiency\textsuperscript{67} and classic IDA in humans are associated with low hepcidin levels\textsuperscript{67} which makes hepcidin a potential marker for detection of IDA in ACD.\textsuperscript{67} However, studies in anemic patients suffering from diseases such as rheumatoid arthritis, inflammatory bowel diseases, cancer, and end-stage renal disease are needed to validate the potency of hepcidin measurements under these conditions.

Third, hepcidin analysis might have a role as a screening, prognostic and monitoring test for hereditary hemochromatosis (HH), provided that abnormalities in liver functions,\textsuperscript{70} inflammation and a short interval between sample collection and phlebotomy are excluded. As shown in Supplemental Table 1, panel B, at presentation urinary hepcidin levels are low or even undetectable in all cases of juvenile hemochromatosis and likely to be moderately decreased or low for the increased iron stores in TFR2 and HFE sequence variants. In our opinion, assessment of hepcidin values might be instrumental in determining the likelihood of candidate genes involved in patients with non-HFE HH. This might reduce the workload and costs of the slow screening procedures for sequence variations in the multiple genes responsible for hemochromatosis.

Fourth, in iron loading anemias such as thalassemia, studies have suggested hepcidin or hepcidin/ferritin index values at the lower end of the reference range to be a result of suppressed hepcidin production due to high and less effective erythropoietic activity.\textsuperscript{69} These findings may be relevant in the search for non-invasive measures of iron burden and improved therapeutic interventions for these often congenital diseases.

Finally, determination of hepcidin levels might prove useful in predicting a response to EPO and iron treatment in patients with chronic disease anemias, as well as in the monitoring of EPO treatment. Until now, predicting how patients will react to EPO treatment is complicated by the co-existence of several factors that contribute to anemia, such as inflammatory activity and liver toxic therapy. Further studies are warranted to determine hepcidin levels in chronic kidney disease, end-stage renal disease, on hemodialysis and EPO and/or iron treatment in relation to iron and inflammatory status and blood counts. These studies will lead to novel diagnostic and improved therapeutic strategies in patients treated with EPO.

**Conclusions**

The regulation of body iron, an essential but also toxic element is strictly controlled by a small peptide hormone hepcidin. This principal liver-produced peptide is characterized by its unique anti-microbial structural and functional properties which places it at the crossroads between innate immunity, host defence and iron metabolism. Body iron levels, inflammation and erythropoietic activity appear to be the main regulators of hepcidin. Recent research has provided new insights into the main regulatory pathways but also into other networks that interact in hepcidin synthesis. Altogether, the body iron homeostasis network with hepcidin at the centre appears to be more complex than was originally thought.
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10. Pigeon C, Ilyin G, Courselaud B, and urine will be essential to translate results obtained from mice and in vitro studies to humans. A wider knowledge of hepcidin regulation will provide us with novel tools for differential diagnosis, therapeutic regimes and monitoring of disorders of iron metabolism. We apologize to all our colleagues whose work we could not cite due to limitations of space.

Authorship and Disclosures

EK designed and wrote the paper; HT, HW and DS co-wrote and revised the paper. The authors reported no potential conflicts of interest.
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