Mini-Review

Current Status of the Measurement of Blood Hepcidin Levels in Chronic Kidney Disease

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Hepcidin is a small defensin-like peptide produced in the liver in response to anemia, hypoxia, or inflammation. In addition to its anti-microbial properties, it has also been found to be a key regulator of iron utilization, providing increased understanding of why chronic kidney disease patients absorb iron poorly from the gut and also why many hemodialysis patients develop functional iron deficiency in the presence of inflammation. Hepcidin synthesis is upregulated in uremia, as in other inflammatory states. The ability to measure hepcidin in biologic fluids has stimulated interest in the potential applicability of this measurement as a more informative marker of iron status than the traditional iron indices such as serum ferritin and transferrin saturation. Until recently, however, the assays for measuring hepcidin have lacked precision, accuracy, and internal validation. Over the last few years, however, several assays have become available that address these limitations. Broadly speaking, these can be divided into radioimmunoassays, ELISAs, and mass spectrometry methods. The purpose of this review is to outline the various assays available at the present time, to critique their advantages and limitations, and to report comparative data in patients with chronic kidney disease. A concern with the immunoassays is that they detect more than biologically active hepcidin-25. Mass spectrometric assays are specific for hepcidin-25 but are labor intensive and require more costly and sophisticated instrumentation. Thus, although mass spectrometry is more accurate, it is less practical for routine clinical use at the present time.

Hepcidin Structure and Synthesis

Hepcidin is a recently discovered small, cysteine-rich cationic peptide that was initially identified in human blood using mass spectrometry (MS) (1). Subsequently, Park et al. (2) isolated urinary hepcidin-25 and hepcidin-20 (truncated N-terminal pentapeptide) using cation exchange chromatography and reverse-phase HPLC. The major site of synthesis for this peptide is the liver hepatocyte, where it is initially encoded as a prepropeptide, although there is some evidence that the kidneys may also be involved (3). The peptide possesses anti-microbial properties, but it has also been found to be a key regulator of iron utilization, binding to the iron exporter protein, ferroportin, and consequently modulating the activity of other proteins involved in iron availability such as divalent metal transporter-1 and transferrin (4–8). Its production is influenced by a variety of stimuli, including anemia, hypoxia, and inflammation. Indeed, IL-6 has been shown to be a potent stimulant of hepcidin synthesis. The peptide is apparently upregulated in uremia, as in other chronic inflammatory states (9–12), and its presence has provided a biologic explanation for why chronic kidney disease (CKD) patients absorb iron poorly from the gut and why many hemodialysis patients develop functional iron deficiency in the presence of inflammation.

Further elucidation of the role of hepcidin in CKD, however, has been hampered over the last few years by the lack of availability of suitable assays to measure the peptide in biologic fluids. Being a small peptide, it is difficult to raise antibodies against it, and the early assays examined hepcidin in urine, which had obvious and significant limitations in CKD patients. More recently, measurements of hepcidin in blood have been developed, and it is possible that, with further refinement, hepcidin measurements could be more informative of iron status in CKD than the traditional markers of iron status such as serum ferritin and transferrin saturation.

Hepcidin Structure and Synthesis

Hepcidin is synthesized in the liver, where it is encoded as an 84-amino acid prepropeptide. The first 24 amino acids of the prepropeptide contain an endoplasmic reticulum targeting signal that is cleaved to produce the 60-residue prohepcidin. Further enzymatic cleavage of prohepcidin produces the bioactive 25-amino acid hepcidin (13,14). The 25-residue active peptide contains eight cysteine residues, constrained by four disulphide bridges. Formation of the four disulphides to produce the biologically active peptide has been particularly problematic, and the assignment of disulphide pairing remains a challenge using proteolysis and MS. Recently, the disulphide pairings have been revised (1–8, 2–4, 3–6, and 5–7) using detailed nuclear
magnetic resonance spectroscopy and reductive alkylation of disulfide pairs (15). This structure results in a distorted β-sheet with a hairpin loop, stabilized by disulfide pairing and hydrogen bonds, giving rise to an amphipathic structure.

The amphipathic structure of hepcidin renders it susceptible to adsorption to surfaces. A recent study using radiolabeled hepcidin has indicated that serum hepcidin is largely bound to α₂-macroglobulin (16). Hepcidin is broken down into smaller fragments that do not seem to be biologically active. Thus, hepcidin assays should take into consideration the presence of truncated hepcidins, the adsorption of the peptide onto surfaces, and the binding of the peptide to plasma proteins.

Hepcidin Assays

Broadly speaking, there are four main types of hepcidin assays available for the detection of this peptide in biologic fluids: RIA, ELISA, a ligand binding assay, and MS. Ideally, all hepcidin assays should satisfy the following minimum criteria: specificity, which is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present; accuracy, which is closeness of the value with the experimental determined value; precision, which is the expression of the closeness of agreement between a series of measurements obtained by multiple sampling under prescribed conditions expressed as a coefficient of variance (CV); detection limit, which is the lowest amount that can be detected but not necessarily quantified; limit of quantitation, which is the lowest amount that can be quantified with suitable precision (<20% CV); and linearity, which is obtaining results that are directly proportional to the concentration of the analyte.

RIA

RIA is a very sensitive technique used to measure concentrations of antigens (e.g., hormones in the blood) without the need for a bioassay. A known quantity of antigen is made radioactive, often by labeling it with an iodine isotope attached to either tyrosine or histidine. This radiolabeled antigen is mixed with a known amount of antibody for that antigen, and as a result, the two compete for the same binding site. Next, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen for antibody binding sites. RIA is extremely sensitive and highly specific. Despite these advantages, the method has been largely superseded by ELISA, where the antigen–antibody reaction is measured using colorimetric signals instead of a radioactive signal. Indeed, many hospitals have abandoned the use of RIAs because of safety concerns regarding the use of radioisotopes.

At the time of writing, there is only one commercially available RIA: the Hepcidin-25 RIA kit (Bachem, UK; http://www.bachem.com). According to the manufacturer, the range for measurement of hepcidin is 0.1 to 13 ng/ml (10 to 1280 pg/tube), with antigen labeled with 125I. The standard is hepcidin-25, showing no cross-reactivity with human angiotensin I, human insulin, and glucagon(1–29), from a variety of species (human, bovine, porcine, rat, mouse, hamster). As far as the authors are aware, there are no published comparisons of the kit with any hepcidin ELISA assays.

Recently, Ashby and colleagues (9,17) published details of an RIA for hepcidin. In this assay, commercially available hepcidin was radioiodinated, and polyclonal rabbit anti-hepcidin antibodies were produced using synthetic hepcidin conjugated to keyhole limpet hemocyanin. Binding was assessed by competition to 125I-labeled hepcidin, separated by a secondary antibody. This assay was reported to be linear over the range 0.6 to 200 ng/L, with a limit of detection of 0.6 ng/ml and an inter-assay precision <10% for the concentration range of 2.8 ng/ml with good recovery. No cross-reactivity was observed with prohepcidin, but there was a 10% cross-reactivity with the biologically inactive fragment, hepcidin-20.

Recently, Swinkels and her colleagues (18) also described a RIA for the detection of hepcidin in human serum. This assay was reported to exhibit a very low detection limit (0.02 ng/ml), low imprecision (4.4 to 6.2%), and good linearity and recovery (range: 81 to 105%). Hepcidin levels of samples of controls and patients with iron deficiency and inflammation showed an excellent correlation with previously described quantitative time-of-flight MS assay (range, 2.5 to 267 ng/ml; \( r = 0.92, P < 0.0001 \) (14). This RIA also exhibits a 10 to 20% cross-reactivity with hepcidin-20 and hepcidin-22.

The above two assays use similar procedures but show a 30-fold difference in detection limit. The only apparent difference between the two assays is that the Swinkels assay incorporates a coating of the plate with anti-rabbit IgG antibody before treatment with the anti-hepcidin antibody.

ELISA

ELISA is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In this method, an unknown amount of antigen is attached to a surface, and a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step, a substrate is added such that the enzyme can generate a detectable signal.

Kulaksiz et al. (3) have developed an ELISA assay for quantitation of prohepcidin in serum. Rabbit anti-hepcidin antibodies for prohepcidin were raised using a synthetic peptide immobilized on a solid support. The peptide was designed to represent an epitope of hepcidin precursor protein (residues 28 to 47, which do not overlap with the hepcidin sequence). N-terminally biotinylated prohepcidin (28–47) was used as a competitor and a signal development tool with streptavidin-peroxidase to facilitate detection. This assay is reported to be highly reproducible, stable, and sensitive; however, the prohepcidin levels failed to show any consistent correlation with known iron storage parameters (serum ferritin, iron, or transferrin saturation [19] or iron absorption [20]). This lack of correlation was confirmed in another study where prohepcidin levels failed to increase in response to induced infection (21). These results suggest that serum prohepcidin concentrations do not reflect iron metabolism changes in iron regulatory disorders in the same way as bioactive hepcidin.
Recently, a competitive ELISA has been developed in which antibodies are raised against hepcidin, immobilized on a solid support, and incubated with samples (22). Biotinylated hepcidin was used as a competitor and a detection tool (Intrinsic LifeSciences; La Jolla, CA), whereas streptavidin-peroxidase facilitates detection. The ELISA standard curve (presented in gravimetric units, ng/well) was not affected by the addition of any of the hepcidin-poor sera obtained from patients with a serum ferritin <10 ng/ml, and no competition was detected when an anti-microbial peptide, protegrin-1, was added. No accuracy or recovery data were presented, but an intra-assay precision of 5 to 19% and interday precision of 12% (with a range of 0 to 44%) with a detection limit of 5.6 ng/ml were reported. A somewhat higher variation was observed at concentrations <31 ng/ml. This method was used to measure hepcidin values of clinical samples in the range 5.6 ng/ml to 4 µg/ml. The quoted detection limit falls in the last quartile of the calibration curve and is prone to error. Examination of the precision of the detection limit of this assay may be closer to 50 ng/ml rather than the quoted value of 5.6 ng/ml, because the authors indicate that there are appreciable serum levels of both hepcidin-20 and hepcidin-22 has also not been reported for this assay. This ELISA assay was recently used to determine blood hepcidin levels in CKD patients, confirming raised plasma hepcidin levels in renal failure compared with normal healthy controls, which is consistent with the notion of uremia being a chronic inflammatory state (23). At the present time, however, there is no indication as to how hepcidin is attached to the carrier protein used to raise the antibodies employed in this method, and how hepcidin is biotinylated, nor how much antibody or biotinylated hepcidin is added to the sample in a typical assay. It would be useful to know the extent of cross-reactivity of the antibodies for prohepcidin, hepcidin, and hepcidin fragments.

Recently, Kollarik and colleagues reported an ELISA assay for hepcidin using a recombinant peptide GA-Hep-FDH6 (recHep ELISA) (24) and polyclonal antibodies raised against this peptide (25). This ELISA assay has a detection range of 11 to 1395 ng/ml, with a detection limit of 5.6 ng/ml. However, the recombinant peptide is appreciably different from the native peptide. The recHep ELISA reports normal control hepcidin levels of 42 ng/ml, lower levels for hemochromatosis (12.6 ng/ml), and higher levels for patients with Hodgkin’s lymphoma (114 ng/ml). The cross-reactivity of hepcidin-20 and hepcidin-22 has not been reported in this investigation.

In a newly developed ELISA assay by DRG (http://www.drg-diagnostics.de), monoclonal antibodies against bioactive hepcidin-25 are used, with no detectable cross-reactivity against prohepcidin, α-fetoprotein, human chorionic gonadotropin, human placental lactogen, and follicle stimulating hormone. According to the product literature, the assay range is 0.9 to 140 ng/ml, with normal control hepcidin levels of 54 ng/ml. This assay is awaiting validation by an independent party.

The detection limit for hepcidin is 0.6 ng/ml in the study by Ashby et al. (9) and 5.6 ng/ml in the study of Ganz et al. (22). In addition, the normal values for hepcidin in both studies are different. According to Ashby et al. (9), the normal range for healthy volunteers is 2 to 56 ng/ml (5th to 95th percentile) with a median of 11 ng/ml. In CKD patients, hepcidin levels ranged from 3 to 153 ng/ml with a median of 26.5 ng/ml, and in hemodialysis patients, hepcidin levels ranged from 25 to 159 ng/ml with a median of 59 ng/ml. Zaritsky et al. (12) reported a median hepcidin value of 270 ng/ml in adult CKD patients and a value of 73 ng/ml in healthy volunteers.

In a recently published international “round robin” study centered on the first steps toward harmonization of hepcidin measurement, each participating laboratory analyzed the same seven samples in triplicate on 4 consecutive days (26). The methods included RIA (9), ELISA (22), and recHep ELISA (24) assays. Although the mean of all of the samples measured by the RIA was 48 ng/ml, the recHep ELISA measured a two-fold increase, whereas the ELISA measured a seven-fold increase in hepcidin. For the between-day precision, the ELISA exhibited the highest CVs. The reasons for these differences are as yet unclear and require further elucidation. All assays conducted in CKD patients show consistently higher plasma levels of hepcidin compared with normal healthy individuals, but the differences in absolute levels between different assays is a concern and requires further clarification. Cross-reactivity is a particular problem with serum hepcidin quantification in patients with end-stage renal failure, where it is established that there are appreciable serum levels of both hepcidin-20 and hepcidin-22 (27).

### Ligand Binding Assay

A functional ligand binding assay has been described by De Domenico et al. (28) in which the ability of human serum to compete with 125I-hepcidin for binding to the l-hepcidin binding domain (HBD) is assayed. They determined the concentration of hepcidin in serum and showed a linear relationship with plasma volume but not concentration. A known quantity of chemically synthesized hepcidin added to serum was quantitatively recovered, but data were not provided. Repeated measurements of hepcidin in sera showed <5% variation. This work reported that healthy men had, in general, higher hepcidin levels than healthy females. The method has not been used to study CKD or dialyzed patients. Insufficient analytical information has been reported for this assay. The stability of the HBD has not been investigated, e.g., does the cysteine residue in the HBD form a disulphide? Can it result in disulphide scrambling with other disulphide-rich peptides? For the recovery experiment, “a known quantity of chemically synthesized hepcidin added to serum was quantitatively recovered (data not shown)”; this experiment needs to be carried out at a minimum of three different concentrations and in triplicate. For precision and accuracy, the authors reported “repeated measurements of hepcidin in sera showed less than 5% variation”; again, insufficient analytical information is provided because both precision and accuracy should be determined for a range of concentrations, and a limit of detection and a limit of quantitation should also be determined. This assay needs to be carefully validated for stability, specificity, accuracy, precision, and recovery.
MS

MS is an analytical technique for the determination of the elemental composition and structure elucidation of small molecules including peptides. The principle of MS consists of ionization of a molecule to generate charged species or molecule fragments and measurement of their mass-to-charge ratios.

Surface Enhanced Laser Desorption/Matrix-Assisted Laser Desorption Ionization

Matrix-assisted laser desorption ionization (MALDI) is a “soft” ionization technique that allows for the sensitive detection of large, nonvolatile, and labile molecules by MS. The excitation of the large excess of matrix by a high intensity laser pulse over a short duration induces desorption and ionization processes of both the matrix and the analyte. The ions thus formed are subsequently extracted and accelerated by a strong electric field into a flight tube of a time-of-flight (TOF) analyzer. The flight time corresponds to the mass-to-charge ratio. Surface enhanced laser desorption (SELDI) is a variant of MALDI where the MS target has incorporated surface chemistry (e.g., cation exchange, immobilized metal affinity capture [IMAC], or reverse phase for sample clean up).

SELDI was the first MS method used for the semiquantitative measurement of hepcidin in urine (29) and plasma (30). Hepcidin determination in the serum of hemodialysis patients was carried out using SELDI (Ciphergen Biosystems, Freemont, CA), with a reported detection limit of 53 ng/ml (30). The intensities of the two peaks corresponding to hepcidin-20 and hepcidin-25 were found to be higher in hemodialysis patients compared with healthy volunteers, and a correlation with serum ferritin was found. The authors also reported that hepcidin-25 levels did not seem to fall after a dialysis session in some of the patients (30).

This semiquantitative method was further explored (31), and to overcome some of these limitations, desAsp-hepcidin (hepcidin-24) was used as an internal standard (32,33). Recovery in analytical procedures involves spiking a blank sample with low, medium, and high concentrations of analyte, followed by analysis to compare experimentally observed values with the expected values. There is no analytical recovery or accuracy reported for this method, but good precision (<10% CVs) is reported with a low limit of quantitation in urine. The methodology adopted by Swinkels et al. (33) uses immobilized metal affinity capture to chelate the amino terminal Cu(II) and Ni(II) binding (ATCUN) motif present on the N-terminus of hepcidin. Their data suggest different binding properties of desAsp-hepcidin and hepcidin-25, with a ratio of 0.7; exactly how much impact the use of desAsp-hepcidin as an internal standard has on the accurate quantitation of hepcidin is unclear. This method has successfully detected physiologic and pathologic changes in serum hepcidin in patients with various disorders of iron homeostasis. Subsequently, the authors have used a MALDI-TOF; this is described below.

Recently, Ward et al. (34) used an isotopically labeled hepcidin as an internal standard for the quantitation of hepcidin using SELDI-TOF MS. No accuracy and recovery data have been presented; however, there is good intraday (<20%) and interday (<20%) precision with this assay. The lowest quantity of internal standard reliably detected in serum was 10 ng/ml, and this study found the average hepcidin concentration in healthy women to be 50 ng/ml. High-resolution MALDI-TOF with a stable isotope-labeled hepcidin internal standard has also been used to analyze hepcidin samples (35,36). However, in this assay, only urine samples were reliably analyzed because serum samples presented difficulties associated with peak resolution. The Swinkels group first reported the analysis of hepcidin using a SELDI and have recently switched to MALDI-TOF (27,37). In this new assay, they still used hepcidin-24 as an internal standard, the merits of which have been discussed above; sample preparation has been carried out using Macro-Prep carboxymethyl support beads, which was first described by Nemeth et al. (5) for the Dot-blot hepcidin assay in urine. The samples were analyzed using MALDI-TOF in the linear mode. This method reports a lower limit of detection of 1.4 ng/ml, with both inter-run and intrarun imprecision <10% and a reference level of serum hepcidin of 11.7 ng/ml with a range of 1.4 to 38.8 ng/ml. In this “improved” method, the MS used the linear mode, and isotopic resolution is not achieved, whereas recent MALDI methods have used isotopic resolution (35,36,38). A major advantage of SELDI, namely the ease of sample preparation, was replaced with a chromatographic step using weak cation exchange media that requires considerable more processing. Good analytical characteristics of accuracy and precision are reported with this assay.

Liquid Chromatography MS/MS Assays

With liquid chromatography (LC) MS/MS, the sample is separated by liquid chromatography and the eluent is analyzed directly. This is in contrast to SELDI and MALDI, where the crystallized sample is ionized as such. Detection is achieved by fragmentation of the precursor ion (which is usually multiply charged) to the product ion. This process is called selected reaction monitoring and is indeed highly selective, providing a considerable increase in sensitivity.

Murphy et al. (39) first reported an LC MS/MS assay for hepcidin where the sample was fractionated using reverse-phase solid phase extraction. The samples were fractionated on a CAPCELL PAK C18 column and analyzed for hepcidin. The method used calcitonin gene-related peptide as an internal standard, which is not ideal because of its differing size and charge. In the validation, rabbit serum was spiked with hepcidin with a recovery of 60% and 90% for the analyte and the internal standard, respectively. This method reports good accuracy and precision with a lower limit of quantitation of 1.0 ng/ml and is associated with an average serum hepcidin concentration of 8 ng/ml for five subjects. Hepcidin has also been analyzed using a similar methodology by Murao et al. (40). This method reports a recovery of 37% from trichloroacetic acid precipitation followed by LC MS/MS. Kobold et al. (41) have used a micro-LC MS/MS to measure hepcidin. Reporting a recovery of 95 to 100% from the assay with a lower limit of quantification of 3.3 ng/ml (CV <20%), data from 10 normal controls yielded a mean hepcidin concentration of 5.6 ng/ml.

Li et al. (42) have developed an LC MS/MS assay for hepcidin determination in serum hepcidin in patients with various disorders of iron metabolism. The measurement of hepcidin in serum of hemodialysis patients was carried out using SELDI (Ciphergen Biosystems, Freemont, CA), with a reported detection limit of 53 ng/ml (30). The intensities of the two peaks corresponding to hepcidin-20 and hepcidin-25 were found to be higher in hemodialysis patients compared with healthy volunteers, and a correlation with serum ferritin was found. The authors also reported that hepcidin-25 levels did not seem to fall after a dialysis session in some of the patients (30).

This semiquantitative method was further explored (31), and to overcome some of these limitations, desAsp-hepcidin (hepcidin-24) was used as an internal standard (32,33). Recovery in analytical procedures involves spiking a blank sample with low, medium, and high concentrations of analyte, followed by analysis to compare experimentally observed values with the expected values. There is no analytical recovery or accuracy reported for this method, but good precision (<10% CVs) is reported with a low limit of quantitation in urine. The methodology adopted by Swinkels et al. (33) uses immobilized metal affinity capture to chelate the amino terminal Cu(II) and Ni(II) binding (ATCUN) motif present on the N-terminus of hepcidin. Their data suggest different binding properties of desAsp-hepcidin and hepcidin-25, with a ratio of 0.7; exactly how much impact the use of desAsp-hepcidin as an internal standard has on the accurate quantitation of hepcidin is unclear. This method has successfully detected physiologic and pathologic changes in serum hepcidin in patients with various disorders of iron homeostasis. Subsequently, the authors have used a MALDI-TOF; this is described below.

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Hepcidin Measurement in CKD Patients

All three types of assays described above have been used to measure blood hepcidin levels in CKD patients (Table 1). The original SELDI-TOF assay described by Tomosugi et al. (30) found hepcidin levels to be elevated in hemodialysis patients compared with normal healthy controls. This has been confirmed by various other groups (9,11,17,22,23,27,37,42,44,45), although the absolute values of hepcidin vary up to 10-fold depending on the assay used. Thus, most assays suggest a normal range for plasma hepcidin to be in the range of 1 to 55 ng/ml, whereas values from hemodialysis patients are in the range of 27 to 158 ng/ml (Table 1). Studies that have also examined nondialysis CKD patients have generally reported normal control values of 50 ng/ml (34) and 3 to 17 ng/ml (32), whereas LC MS/MS has reported normal control values of 5.6, 8.4, 10, and 11 ng/ml (39,40,42,44). It is unclear whether some of the methods are measuring other analytes as well as hepcidin or if methods are measuring only a proportion of the hepcidin present. The international “round robin” on the first steps toward the harmonization of hepcidin measurement has also highlighted this difference in hepcidin measurements (26). Great care needs to be exercised in correlating hepcidin concentrations determined using different methods. A stringent standardization in hepcidin measurement is urgently required. Furthermore, hepcidin is a “sticky” peptide that will bind to hydrophobic surfaces including plasma proteins; therefore, all measurements carried out using the immunoassays described above will probably only measure the circulating hepcidin. It is important to establish whether the various assays are detecting free, bound, or total hepcidin and what is the more appropriate measurement. For example, endogenous hepcidin binding protein(s) may be present in serum and plasma (α2-macroglobulin) and may markedly influence assay performance. Their concentration and the hepcidin-binding characteristics may be subject to both genetic and acquired influence (44,47). Furthermore, hepcidin is a small peptide and is expected to be readily cleared from the kidney; however, because it is specifically bound to α2-macroglobulin (16), its clearance is minimized. Recent studies have reported that circulating hepcidin only accounts for 10% of total hepcidin, the remainder being bound to α2-macroglobulin (16).

Conclusions

Ever since the discovery of hepcidin nearly 10 years ago, various groups of scientists have attempted to develop suitable assays for the measurement of this peptide in biologic fluids. Early attempts were thwarted by the difficulty of producing antibodies against this small peptide, and although this has now been achieved, the concern now is that the assay is detecting other forms of hepcidin than the biologically active hepcidin-25. Nephrologists may quite rightly have a sense of déja vu, recalling that the early assays for parathyroid hormone detected fragments of PTH rather than the intact molecule. The
### Table 1. Details of blood hepcidin assays used to measure levels in CKD patients

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of Assay</th>
<th>Reference Levels</th>
<th>CKD Patients</th>
<th>Findings/Comments</th>
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<tbody>
<tr>
<td>Tomosugi et al. 2006</td>
<td>SELDI-TOF</td>
<td>16 normals (25 ± 9 arbitrary units)</td>
<td>40 HD</td>
<td>Higher in HD than in controls</td>
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<tr>
<td>Kato et al. 2008</td>
<td>SELDI-TOF</td>
<td>25 HD (59 ± 9 arbitrary units)</td>
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<td>Hepcidin cleared by HD</td>
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<td></td>
<td>(semiquantitative)</td>
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<td>No difference between ESA responders and nonresponders; no effect of type of</td>
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<td>dialyzers on hepcidin</td>
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<td>Ashby et al. 2009</td>
<td>RIA</td>
<td>64 normals (mean 10.8 ng/ml (range 2 to 55 ng/ml</td>
<td>44 CKD (mean 26.5 ng/ml (range 3.1 to 153 ng/ml)</td>
<td>CKD and HD had no clear diurnal pattern of hepcidin in contrast to healthy</td>
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<td>(semiquantitative)</td>
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<td>controls</td>
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<td>Hepcidin not reduced by HD (n = 6)</td>
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<td>Heparlin increased after intravenous iron (n = 4)</td>
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<tr>
<td>Busbridge et al. 2009</td>
<td>RIA</td>
<td>47 normals (mean 13 ng/ml (range 1.1 to 55.3 ng/ml)</td>
<td>37 CKD (mean 26 ng/ml (range 0.8 to 130 ng/ml)</td>
<td>Heparlin increased after IV iron (CKD n = 4) from 18.1 ± 9.6 to 59.3 ± 18.6 ng/ml after 24 hours</td>
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<tr>
<td>Zaritsky et al. 2009</td>
<td>Competitive ELISA</td>
<td>20 healthy children (mean 25.3 ng/ml)</td>
<td>48 PaedCKD2-4 (mean 127.3 ng/ml)</td>
<td>Responders to ESA had higher hepcidin than healthy controls, but no difference</td>
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<td>24 healthy adults (mean 72.9 ng/ml)</td>
<td>32 AdultCKD2-4 (mean 269.9 ng/ml)</td>
<td>between responders and non-responders</td>
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<td>26 PaedHD (mean 652.4 ng/ml)</td>
<td>Heparlin/ferritin ratio was lower in 25 HD patients with HFE mutations,</td>
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<td>compared with 40 HD patients without</td>
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<td>No difference between low and high-flux</td>
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<td>Hepcidin cleared by HD session</td>
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<td>Costa et al. 2009</td>
<td>WCX–MALDI_TOF</td>
<td>17 normals (mean 6.4 ng/ml (range 0 to 9 1 ng/ml)</td>
<td>33 HD (mean 24.5 ng/ml (range 10.6 to 39.5 ng/ml)</td>
<td>Responders to ESA had higher hepcidin than healthy controls, but no difference</td>
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<td>(37)</td>
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<td>between responders and non-responders</td>
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<td>Valenti et al. 2009</td>
<td>SELDI-TOF</td>
<td>57 normals (mean 12.9 ng/ml (range 8.4 to 19.6 ng/ml)</td>
<td>65 HD (mean 29.6 ng/ml (range 7.5 to 65.9 ng/ml)</td>
<td>Responders to ESA had higher hepcidin than healthy controls, but no difference</td>
</tr>
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<td></td>
<td>(48)</td>
<td></td>
<td></td>
<td>between responders and non-responders</td>
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<td></td>
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<td></td>
<td></td>
<td>Heparlin/ferritin ratio was lower in 25 HD patients with HFE mutations,</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>compared with 40 HD patients without</td>
</tr>
<tr>
<td>Weiss et al. 2009</td>
<td>LC-MS/MS</td>
<td>20 HD (45.5 ± 6.4 ng/ml)</td>
<td></td>
<td>No difference between low and high-flux</td>
</tr>
<tr>
<td></td>
<td>(46)</td>
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<td>dialyzerers</td>
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“round robin” exercise of comparing measurements of hepcidin among different assays worldwide has highlighted this problem, with a 10-fold variation between the more sensitive mass spectrometric methods and the immunoassays, such as the recently published ELISA. Although these limitations do not preclude comparisons of samples from CKD patients and healthy controls, they do make it difficult to determine the accurate reference range for plasma hepcidin levels in CKD, and further attempts should be made to refine the methodology of the different assays. If this can be achieved, it is possible that measurement of serum hepcidin may be more informative than the traditional markers of iron status that have been used over the last two decades (ferritin, transferrin saturation, hypochromic red cells, reticulocyte hemoglobin content), and it may even be that future strategies for managing anemia in CKD patients may be directed at reducing plasma levels of this peptide.

Disclosures
The authors of this review have been using an ELISA for prohepcidin (J.M.), both an ELISA and an RIA for hepcidin measurement (J.M.), and are actively pursuing the development of mass spectrometric assays for hepcidin (S.B., R.C.H., and I.C.M.). There are no other disclosures to report.

References

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<td>Reference</td>
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<td>Li et al. 2009 (42)</td>
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<td>Peters et al. 2010</td>
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<td>Bansal et al. 2010</td>
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