Hepcidin—A Potential Novel Biomarker for Iron Status in Chronic Kidney Disease

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Background and objectives: Hepcidin is a key regulator of iron homeostasis, but its study in the setting of chronic kidney disease (CKD) has been hampered by the lack of validated serum assays.

Design, setting, participants, & measurements: This study reports the first measurements of bioactive serum hepcidin using a novel competitive ELISA in 48 pediatric (PCKD2–4) and 32 adult (ACKD2–4) patients with stages 2 to 4 CKD along with 26 pediatric patients with stage 5 CKD (PCKD5D) on peritoneal dialysis.

Results: When compared with their respective controls (pediatric median = 25.3 ng/ml, adult = 72.9 ng/ml), hepcidin was significantly increased in PCKD2–4 (127.3 ng/ml), ACKD2–4 (269.9 ng/ml), and PCKD5D (652.4 ng/ml). Multivariate regression analysis was used to assess the relationship between hepcidin and indicators of anemia, iron status, inflammation, and renal function. In PCKD2–4 ($R^2 = 0.57$), only ferritin correlated with hepcidin. In ACKD2–4 ($R^2 = 0.78$), ferritin and soluble transferrin receptor were associated with hepcidin, whereas GFR was inversely correlated. In PCKD5D ($R^2 = 0.52$), percent iron saturation and ferritin were predictors of hepcidin. In a multivariate analysis that incorporated all three groups ($R^2 = 0.6$), hepcidin was predicted by ferritin, C-reactive protein, and whether the patient had stage 5D *versus* stages 2 to 4 CKD.

Conclusions: These findings suggest that increased hepcidin across the spectrum of CKD may contribute to abnormal iron regulation and erythropoiesis and may be a novel biomarker of iron status and erythropoietin resistance.

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Recombinant erythropoietin (rhEPO) has transformed anemia therapy in patients with chronic kidney disease (CKD). However, rhEPO resistance, often associated with iron deficiency and inflammation, remains a challenging problem (1–4). Current available iron indices do not reliably identify iron-restricted erythropoiesis, often a sequela of inflammation, or those patients who would likely benefit from parenteral iron therapy (5–7). To address these issues, it is crucial to understand the molecular mechanisms that link inflammation, iron balance, and erythropoiesis.

Hepcidin, an acute phase reactant protein produced in the liver, is a recently discovered key regulator of iron homeostasis. Hepcidin inhibits intestinal iron absorption and iron release from macrophages and hepatocytes (8). Because hepcidin production is increased by inflammation, and high hepcidin concentrations limit iron availability for erythropoiesis, hepcidin likely plays a major role in the anemia of inflammation and rhEPO resistance.

Due to the previous absence of an accurate serum assay, most studies of hepcidin in humans have been performed using a urinary assay. Because such an assay may not reliably reflect serum hepcidin levels in patients with CKD, previous studies have instead attempted to measure the serum levels of prohepcidin, the peptide precursor of hepcidin (9–11). However, these studies have been difficult to interpret because the relationship between prohepcidin, hepcidin, and iron parameters remains unclear (12–16). Mass spectrometry is capable of measuring serum hepcidin and was used to detect a positive correlation between serum hepcidin and ferritin levels in CKD (10,17), but this technique is limited by its semiquantitative nature and requirement for equipment that is not widely available.

Because of its renal elimination (18,19) and regulation by inflammation (20–23), it is possible that progressive renal insufficiency leads to altered hepcidin metabolism, subsequently affecting enteric absorption of iron and the availability of iron stores. In this study, using a novel assay, we present the first quantitative measurements of bioactive serum hepcidin in both pediatric and adult patients across the spectrum of CKD.

Materials and Methods
Patient Criteria

The patient population was comprised of children and adults receiving outpatient care for CKD stages 2–5D as defined by the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines (24) at the Ronald Reagan University of California–Los Angeles (UCLA) Medical Center. The UCLA Institutional Review Board approved this study, and all patients/parents gave informed consent to participate. Pediatric CKD stage 5D (PCKD5D) patients had received at least 3 mo of maintenance automated peritoneal dialysis with a fill volume of 40 cc/kg of

patient's body weight. The average (±SD) weekly Kt/V was 2.1 ± 0.5. Patients receiving rhEPO and iron supplementation were enrolled provided that the dosages of each had been stable for at least 4 wk and all rhEPO supplements were in the form of recombinant epoetin alfa (Amgen). None of the patients received parenteral iron 4 wk before enrollment. In the pediatric CKD 2 to 4 (PCKD2–4) group, 12 patients received both oral iron and rhEPO, 8 patients were prescribed oral iron alone, and 4 patients were administered rhEPO alone. In the adult CKD 2 to 4 (ACKD2–4) group, 4 patients received rhEPO and none were receiving oral iron. Every PCKD5D patient received supplemental oral iron and rhEPO with a 3-mo average (±SD) rhEPO dose of 283 ± 235 units/kg per wk.

Exclusion criteria were (1) previously diagnosed nonrenal cause of anemia other than iron deficiency; (2) evidence of active or occult bleeding, (3) blood transfusion within the past 4 mo; (4) history of malignancy, end-stage liver disease, or chronic hypoxia; and (5) recent hospitalization or infection requiring antibiotics within the past 4 wk.

The control groups consisted of 20 healthy children (8 boys, 12 girls) aged 14.4 ± 4 yr and 24 healthy adults (12 men, 12 women) aged 28.4 ± 6.6 yr.

Data Collection

Demographic data collection included age, sex, cause of CKD, past medical history, and medication history. A one-time blood sample was collected for markers of renal function (creatinine), erythropoiesis (hemoglobin and soluble transferrin receptor), iron status (percent iron saturation and ferritin), inflammation [erythrocyte sedimentation rate and high-sensitivity C-reactive protein (CardioPhase hsCRP—Dade Behring, Inc.)], and renal osteodystrophy (intact parathyroid hormone via first generation Immutopics assay—San Clemente, CA). A simultaneous spot urine sample was collected for measurement of total protein to creatinine ratio in the PCKD2–4 and ACKD2–4 groups. GFR was calculated using the Schwartz equation for patients less than 18 yr of age and the Modification of Diet in Renal Disease equation for those 18 yr or older (25,26).

Serum Hepcidin Measurement

Quantitative measurement of bioactive hepcidin in serum was carried out using a sensitive competitive ELISA (27). Briefly, samples were diluted appropriately, mixed with a biotinylated hepcidin analog (tracer), and added to 96-well microtiter plates coated with rabbit anti-hepcidin antibodies for 2 h. After binding, wells were washed three times with 0.05% Tween 20 in tris-buffered saline (pH 8), and horseradish peroxidase-avidin secondary reagent (1 ng/μl) was added and allowed to bind for 1 h. Wells were washed three times with 0.05% Tween 20 in tris-buffered saline, tetramethylbenzidine substrate was added, and the reactions were stopped with 1 N sulfuric acid. Optical density of reactions at 450 nm was determined on a Beckman Coulter DTX 880 detector. Sample concentrations were determined using Prism curve-fitting software by comparison to a standard curve generated using synthetic hepcidin. Synthetic hepcidin standards were produced by Bachem Biosciences, Inc. (King of Prussia, PA) and validated using HPLC, peptide sequencing, mass spectroscopy, and bioactivity studies conducted in HEK293 cells overexpressing ferroportin-green fluorescent protein.

Statistical Methods

Study variables were summarized using mean and SD. Because of its non-normal distribution, hepcidin values were presented as medians. Log transformation was applied to variables with non-normal distribution. The differences in biochemical measurements between patient groups were compared using ANOVA. Multiple linear regression models were developed to investigate the association of biochemical parameters with hepcidin. Variable selection was done by the LASSO (Least Absolute Shrinkage and Selection Operator) method. All tests were two-sided with significance level of 0.05 and all analyses were performed using SAS statistical software.

Results

Patient Demographics and Biochemical Characteristics

Demographic data for the 48 PCKD2–4, 32 ACKD2–4, and 26 PCKD5D patients are listed in Table 1. There was no significant age difference between the two groups of pediatric patients. The major etiology of pediatric CKD was GN or renal dysplasia, whereas hypertension was the predominant diagnosis in the ACKD2–4 group.

Biochemical markers of renal function (GFR and urine protein to creatinine ratio), erythropoiesis [hemoglobin (Hgb) and soluble transferrin receptor (sTFR)], iron status (percent iron saturation and ferritin), inflammation [erythrocyte sedimentation rate (ESR) and high-sensitivity C-reactive protein (hs-CRP)], and intact parathyroid hormone (iPTH) are presented in Table 2. Because of the increased use of rhEPO, the PCKD5D group had a higher Hgb than PCKD2–4 and ACKD2–4. In addition, the PCKD5D group had a higher percent iron saturation, ferritin, and iPTH. The degree of proteinuria was greater in PCKD2–4 when compared with ACKD2–4, whereas the ACKD2–4 group had the highest levels of inflammation as determined by ESR and hs-CRP.

Serum Hepcidin in CKD Stages 2 to 5

Serum hepcidin levels in all three groups were compared with those of healthy controls (Figure 1). Hepcidin levels were highest in the PCKD5D group, followed by ACKD2–4 and PCKD2–4. When compared with healthy pediatric controls, hepcidin levels were increased in PCKD2–4 ($P < 0.001$) and PCKD5D ($P < 0.001$). Similarly, when compared with healthy adult controls, hepcidin levels were increased in ACKD2–4

Table 1. Patient demographics

Demographic	PCKD2-4[a]	ACKD2-4	PCKD5D
N (men/women)	48 (28/20)	32 (14/18)	26 (11/15)
Age (yr)	13.9 ± 5.4	64.3 ± 16.9	15.4 ± 4.4
Diagnosis (N)[b]			
reflux/dysplasia	12	–	11
FSGS	7	–	5
GN	15	–	6
HTN	–	15	–
HTN + DM	–	7	–
PKD	–	3	–
other	14	7	4

[a]PCKD2-4, pediatric chronic kidney disease (CKD) stages 2 to 4 group; ACKD2-4, adult CKD stages 2 to 4 group; PCKD5D, pediatric CKD stage 5D group.
[b]FSGS, focal segmental glomerulosclerosis; HTN, hypertension; HTN + DM, hypertension and diabetes mellitus; PKD, polycystic kidney disease.

Table 2. Biochemical markers

Marker[a]	PCKD2-4 (mean ± SD)	ACKD2-4 (mean ± SD)	PCKD5D (mean ± SD)
GFR (ml/min/1.73m^2)	43.8 ± 26.2	34.3 ± 14.8	–
UPCR (mg/mg)	7.1 ± 28.7	0.9 ± 2[b]	–
Hgb (g/dl)	12.1 ± 1.9	12.2 ± 1.4	13.3 ± 1.7[b,c]
sTFR (nmol/L)	27.1 ± 7.5	25.1 ± 12.2	22.9 ± 14[b]
Percent iron saturation	22.2 ± 13.6	23.2 ± 8.6	37.7 ± 23[b,c]
Ferritin (ng/ml)	149.3 ± 455	152.9 ± 137	321 ± 303[b,c]
ESR (mm/h)	25.8 ± 19.9	36.4 ± 23.2[b,d]	28.7 ± 24
hs-CRP (mg/L)	4.3 ± 12.6	5.5 ± 6.1[b,d]	2 ± 3.8
iPTH (pg/ml)	139 ± 143.6	107.6 ± 74.6	824 ± 721[b,c]

[a]UPCR, urine protein to creatinine ratio; Hgb, hemoglobin; sTFR, soluble transferrin receptor; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein; iPTH, intact parathyroid hormone.
[b]$P < 0.05$ *versus* PCKD2-4.
[c]$P < 0.05$ *versus* ACKD2-4.
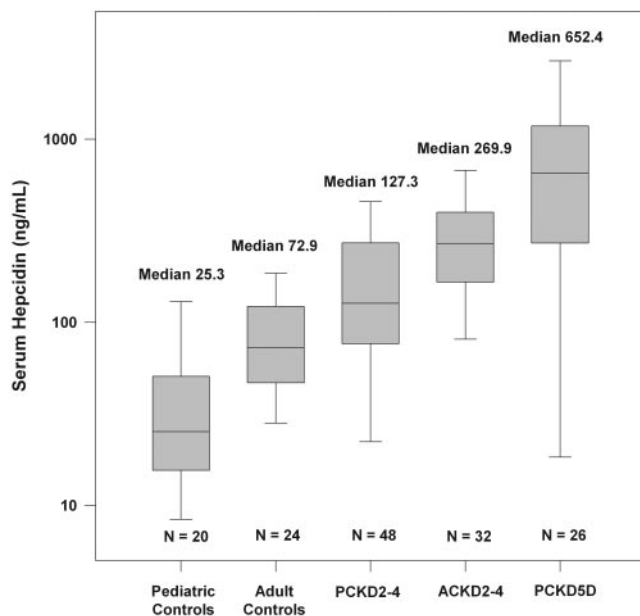[d]$P < 0.05$ *versus* PCKD5D.

Figure 1. Serum hepcidin across chronic kidney disease (CKD) stages. Box plots represent second quartile, median, and third quartile of each group. Error bars denote the 10th and 90th percentile. Hepcidin levels in each group of CKD patients were significantly elevated compared with respective age controls by ANOVA analysis. Additionally, each group of CKD patients had significantly different hepcidin levels (see text).

($P < 0.001$). PCKD5D and ACKD2–4 groups had serum hepcidin concentrations higher than in PCKD2–4 ($P < 0.001$ and $P < 0.013$, respectively); PCKD5D serum hepcidin was elevated compared with ACKD2–4 ($P < 0.004$).

To assess the association between serum hepcidin levels and age, GFR, urine protein to creatinine ratio, Hgb, sTFR, percent iron saturation, ferritin, ESR, hs-CRP, and iPTH in each group, three multivariate regression models were developed (Table 3a). In PCKD2–4, ferritin was the only variable in the model that correlated with serum hepcidin. In the ACKD2–4 model, ferritin and sTFR were directly associated with serum hepcidin, whereas GFR was inversely correlated. A similar multivariate model in PCKD5D that also included the average rhEPO dose as a dependent variable selected percent iron saturation and ferritin as predictors for serum hepcidin

A multivariate regression model was also developed to assess the relationship between serum hepcidin and age, Hgb, sTFR, percent iron saturation, ferritin, average rhEPO dose, ESR, hs-CRP, and iPTH across all CKD stages (Table 3b). Hepcidin was predicted by serum ferritin and hs-CRP. In addition, hepcidin was predicted by whether the patient had stage 5D CKD *versus* stages 2 to 4 CKD.

Discussion

Using a novel validated assay (27), we report the first quantitative measurements of bioactive serum hepcidin in pediatric and adult nondialysis CKD as well as pediatric peritoneal dialysis patients. In this study, we have demonstrated that serum hepcidin is progressively elevated across the spectrum of CKD. As expected from studies in other populations, the elevation of hepcidin appears multifactorial, particularly given its known regulation by iron stores, erythropoiesis, and inflammation (8).

A unique finding in this study was the inverse association between GFR and hepcidin in the ACKD2–4 group. Hepcidin is excreted in urine and metabolized by the kidney (18,19,28). The impairment of one or both of these processes may cause hepcidin accumulation as GFR decreases. It remains possible that the inverse relationship of GFR and hepcidin is a reflection of the known association of CKD and inflammation (1–4); however, when ESR and hs-CRP were included as covariates in our multivariate analysis, the relationship persisted. Although hepcidin levels in the PCKD2–4 group did not correlate with GFR, potential confounders as compared with ACKD2–4 would include increased degree of proteinuria and more frequent iron and rhEPO therapy administration. In addition, the etiology of

Table 3a. Single group multivariate analysis *versus* hepcidin

Variable	PCKD2-4 ($R^2 = 0.57$)		ACKD2-4 ($R^2 = 0.78$)		PCKD5D ($R^2 = 0.52$)	
	$\beta \pm SE$	P	$\beta \pm SE$	P	$\beta \pm SE$	P
Ferritin	0.76 ± 0.11	0.001	0.7 ± 0.09	0.001	0.82 ± 0.15	0.001
Percent iron saturation	–	–	–	–	0.68 ± 0.31	0.02
sTFR	–	–	0.76 ± 0.18	0.001	–	–
GFR	–	–	-0.01 ± 0.005	0.04	–	–

Table 3b. Combined group multivariate analysis *versus* hepcidin

Variable	CKD2-5 ($R^2 = 0.6$)	
	$\beta \pm SE$	P
Ferritin	0.74 ± 0.06	0.001
hs-CRP	0.13 ± 0.05	0.006
PCKD2-4	-0.61 ± 0.18	0.001[a]
ACKD2-4	-0.67 ± 0.18	0.001[a]

[a]*versus* PCKD5D.

renal disease in the ACKD2–4 group was predominantly due to classically noninflammatory causes such as hypertension, diabetes, and polycystic kidney disease. In the PCKD2–4 group, the higher prevalence of chronic GN and the associated inflammatory state may disrupt this association, but further studies of hepcidin metabolism in the setting of renal disease are needed.

The highest levels of hepcidin were observed in the PCKD5D group. Some elevation of hepcidin in this group would be expected given more intensive iron therapy reflected by higher average ferritin and percent iron saturation. Previous studies in populations with normal renal function have shown that oral or parenteral iron loading induces hepcidin production (8,21,29). Elevated levels of hepcidin were present in the PCKD5D group despite the increased use of rhEPO to stimulate erythropoiesis, which typically inhibits hepcidin production (30). Finally, in the multivariate model that included all CKD stages, hepcidin was predicted by whether the patient had stage 5D CKD *versus* stages 2 to 4 CKD. Although further studies are warranted, this finding adds additional support to the concept that worsening renal insufficiency leads to increased hepcidin levels.

In all three CKD populations, a strong correlation was observed between hepcidin and markers of iron status by multivariate analysis, especially with ferritin, the primary storage molecule for cellular iron and a marker of tissue iron stores (5,6). This is consistent with numerous studies in non-CKD populations that have documented a positive correlation between ferritin and hepcidin (8,31,32). In the setting of CKD, the direct relationship with ferritin may represent a protective effect of hepcidin against systemic iron overload. As alluded to earlier, this negative feedback system may be particularly pertinent in PCKD5D because this group of patients had the highest iron indices. In contrast to PCKD2–4 or ACKD2–4 groups, all patients in the PCKD5D group were treated with supple-

mental oral iron and many had received past courses of parenteral iron as recommended by current K/DOQI guidelines (33). This finding may explain why hepcidin remained directly correlated with percent iron saturation in addition to ferritin in the multivariate model of PCKD5D. Our results mirror prior studies using mass spectrometry that report hepcidin to be elevated and correlated with ferritin in hemodialysis patients (10,17).

Although there were no correlations within individual CKD groups between hepcidin and either ESR or hs-CRP, the combined multivariate model did reveal that hepcidin correlated with hs-CRP, an association not found by Kato *et al.* in hemodialysis patients (10). It is known that hepcidin synthesis is induced by inflammation, a process that appears mediated by IL-6 (20–23). Given that CKD is considered an inflammatory state, this positive correlation is expected. The current association may be lessened by our patient selection, because the study protocol excluded patients with active illness or infection. It remains to be seen if in other CKD patients there will be a more robust correlation between inflammation and hepcidin, particularly in patients undergoing hemodialysis.

Our findings are in agreement with a recent report by Ashby *et al.* of serum hepcidin levels in adult CKD and hemodialysis patients using a radio-immunoassay (34). Similar to our results, CKD and dialysis-dependent groups had significantly increased hepcidin levels, with hepcidin inversely correlating with GFR. However, although hepcidin correlated with ferritin in CKD, that association was lost in hemodialysis patients. In addition, Ashby *et al.* found no association between hepcidin and inflammatory markers, although in hemodialysis patients using a multivariate model adjusted for rhEPO and Hgb there was a trend for hepcidin to correlate with IL-6 levels ($P = 0.054$).

Two major types of hepcidin assays are becoming available. In the first, hepcidin peptides can be detected and measured by mass spectrometry, usually after a chromatographic or selective adsorption purification step. Internal standards are used to improve the accuracy of this type of assay. The second type of assay, used in this study and the study by Ashby *et al.* (34), uses an anti-hepcidin antibody in a competitive binding assay between a radiolabeled or tagged hepcidin and the sample. Although the two types of assays correlate extremely well (unpublished data), the absolute values reported by the assays vary by as much as ten-fold. The reason for this discrepancy may include hepcidin-binding factors in sera and urine, differing hepcidin standards and their state of aggregation, and the tendency of hepcidin to adsorb to assay surfaces. Provided that

a suitable reference population is used for each study, these factors do not affect the conclusions reached. Efforts are underway to resolve these differences and to provide technical standardization of the hepcidin assays.

In summary, the increased hepcidin levels seen across the spectrum of CKD have important implications for anemia management in CKD. As renal function worsens there is an increased need for rhEPO administration along with either supplemental oral or parenteral iron. In CKD, increased inflammation and possibly decreased clearance of hepcidin can lead to higher serum hepcidin levels, further contributing to iron-restricted erythropoiesis and rhEPO resistance. Thus high hepcidin levels could predict the need for parenteral iron to help overcome hepcidin-mediated iron-restricted erythropoiesis and the need for relatively higher rhEPO doses to suppress hepcidin production. Conversely, patients with low hepcidin would be expected to respond better to oral iron. If so, hepcidin concentrations may become a unique biomarker to guide iron therapy in CKD.

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Disclosures

Dr. Tomas Ganz and Dr. Elizabeta Nemeth are co-founders and officers of Intrinsic LifeSciences, LLC. Dr. Mark Westerman is a shareholder and officer of Intrinsic LifeSciences, LLC. Dr. Gordana Olbina is a shareholder of Intrinsic LifeSciences, LLC. Dr. Allen Nissenson is a member of the scientific advisory board for Intrinsic LifeSciences, LLC.

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