Impact of hepcidin, interleukin 6, and other inflammatory markers with respect to erythropoietin on anemia in chronic hemodialysis patients

Ihab A. Ibrahim[a], Usama M. Mohamad[a], Hatem A. Darweesh[a],
Amal M. Rashad[b]

[a]Departments of Internal Medicine, Division of Nephrology, [b]Department of Biochemistry; Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence to Ihab Abdelrahman Ibrahim, BSc, MD, Departments of Internal Medicine, Division of Nephrology, Faculty of Medicine, Cairo University, 12918 Cairo, Egypt
Tel: 38037506; Fax: (2)38247220;
email:ihababdelrahman@yahoo.com

Background/objective
Hepcidin is a peptide hormone produced by the liver and appears to be the master regulator of iron homeostasis. This peptide is upregulated in inflammatory conditions, including uremia. Hepcidin functions to regulate (inhibit) iron transport across the gut mucosa, thereby preventing excess iron absorption and maintaining normal iron levels within the body. In this study, we aimed to investigate hepcidin levels and their relationship with the parameters of iron status, inflammation, anemia therapy, and parameters of dialysis efficiency in hemodialysis patients.

Patients and methods
Plasma hepcidin-25, inflammatory markers (high-sensitivity C-reactive protein and interleukin 6), and peripheral iron indices (serum iron, total iron-binding capacity, transferrin saturation and serum ferritin) were measured before hemodialysis in 40 end-stage renal disease (ESRD) patients treated with regular hemodialysis in a single dialysis unit as well as in 20 healthy individuals matched for age and sex serving as the control group.

Results
Plasma levels of hepcidin-25 were significantly higher in hemodialysis patients compared with controls. In a simple correlation analysis, plasma hepcidin levels were positively correlated with ferritin, transferrin saturation, CRP, and interleukin 6; however, it was negatively correlated with hemoglobin, dose of epoitin-α, and dose of iron.

Conclusion
Serum hepcidin levels were associated with iron status and inflammation in maintenance hemodialysis patients, and the high hepcidin serum levels, found in hemodialysis (HD) patients, are dependent on the magnitude of the inflammatory process and on recombinant human erythropoietin doses. Hepcidin and its regulatory pathways are potential therapeutic targets, which could lead to effective treatment of anemia in chronic hemodialysis.

Keywords:
anemia, hemodialysis, hepcidin, inflammation, iron status

Introduction

Anemia is a common complication in maintenance hemodialysis patients and contributes to reduced quality of life [1]. Despite the great success of recombinant human erythropoietin (EPO) in clinical practice for the treatment of anemia in dialysis patients [chronic kidney disease (CKD) patients], resistance to this therapy is ~10–20% [2,3].

Hepcidin is a small defensin-like peptide produced largely by the liver but also by other cells, such as the macrophage and adipocyte [4]. In addition to its antimicrobial properties [5], it is the master regulator of iron metabolism, controlling the amount of dietary iron absorbed from the duodenum and also the release of iron from cells in the reticuloendothelial system (Kupffer cells, splenic macrophages, etc.) [6–8]. At a molecular level, hepcidin binds to the main iron exporter protein ferroportin, which controls iron efflux from duodenal enterocytes, hepatocytes, and macrophages suppressing iron absorption from the intestine and stimulating iron retention in macrophages and hepatocytes [9]. The key role of hepcidin in iron homeostasis and its disorders suggests that its assay in blood or urine could prove useful for the diagnosis and monitoring of iron disorders, and therefore potentially be used as a clinical marker to optimize treatment approaches [10,11].

Hepcidin levels are regulated in response to iron, erythropoietic demand, hypoxia, and inflammatory signals [7]. Iron administration upregulates hepcidin, thereby providing a feedback mechanism to limit further iron absorption, whereas anemia, iron deficiency, and hypoxia inhibit hepcidin, increasing iron availability for erythropoiesis. Hepcidin is also induced by inflammation, which is believed to be part of the host defense mechanism to fight infection and cancer by limiting iron availability to the bone marrow [6,8].

The purpose of the present cross-sectional study was to investigate hepcidin levels in maintenance hemodialysis patients and their relationship with the parameters of iron status, inflammation, anemia therapy, and parameters of dialysis efficiency.

Patients and methods
Participants and research design
Eligibility

All patients were already on maintenance hemodialysis. The inclusion criteria for the studied patients were:
(i) Chronic renal failure from any cause,
(ii) Age more than 18 years and less than 70 years,
(iii) Both sexes were involved,
(iv) Dialysis duration more than 6 months, and
(v) All patients treated by standard bicarbonate dialysis for 4 h, three times a week.

Exclusion criteria were as follows: patients with clinical signs of active neoplasm, acute infections, acute or chronic inflammatory disease, severe liver or lung disease, evidence of blood loss (gastrointestinal bleeding, trauma, etc.), patients using immunosuppressive agents, and patients unable or unwilling to participate in the study.

Study design

This cross-sectional single-center study was performed in a cohort of 40 clinically stable maintenance hemodialysis patients [20 men, 20 women; age (mean ± SD) 45.3 ± 13.17 years] as well as in 20 age-matched healthy control individuals [11 men, nine women; age (mean ± SD) 40.55 ± 8.92 years]. All patients were recruited from the Dialysis Unit in Kasr El-Aini Hospital. Control individuals were age and sex matched and had no abnormal clinical or laboratory findings. All patients were on regular hemodialysis for 4 h a day three times a week for a mean duration of 7.60 ± 4.098 years. All patients had a native arterial–venous fistula. Blood flow rate was 250–400 ml/min and the bicarbonate dialysis fluid flow rate was 500–800 ml/min. Dialysis efficiency [single pool Kt/V (spKt/V)] and ultrafiltration (UF) volume (mean of the last three recorded values) were retrieved from patients' files. Epoitin-α and iron Saccharate (Ferosac, manufactured by SPIMACO, Al-Qassim Pharmaceutical Plant, Saudi Arabia) were prescribed according to the NKF/KDOQI guidelines [2] for anemia therapy.

Hemoglobin was measured monthly, and transferrin saturation (TSAT) index and ferritin were measured every 3 months. The target was to reach a hemoglobin value of at least 10.5 g/dl in more than 85% of patients. None of the patients received parenteral iron 10 days before enrollment. The last three epoitin and iron doses were recorded, and mean values were used in the analysis.

Study protocol and assessments

Protocol determinations were all carried out on the same day and included height, body weight, BMI (weight in kg/squared height in m²), and systolic and diastolic blood pressure. All clinical examinations and evaluations were conducted under fasting conditions.

The quality of dialysis was assessed during the study period by calculating Kt/V for all the patients. Kt/V is defined as the dialyzer clearance of urea and was calculated according to the Daugirdas second generation formula [12].

Blood sampling was taken just before hemodialysis. Fasting blood samples (12 h) were obtained from controls in the morning as well as from hemodialysis patients before the first dialysis session of the midweek (mean Kt/V, a marker of dialysis adequacy, was 1.47 ± 0.3).

Biochemical assessments
Sample collection and handling

Fasting blood samples (12 h) for laboratory analysis were collected on the same day as the examination of the patients. Blood samples were collected in EDTA tubes and centrifuged at 2000g at 2–8°C for 10 min within 30 min of sampling. The plasma was stored in aliquots at −70°C. Repeated freeze–thaw cycles were avoided.

Routine measurements

Routine laboratory measurements of serum levels of albumin (bromocresol green method) [13], calcium (O-cresolphthalein direct method) [14], phosphorus (ammonium molybdate method) [15], triglyceride [16], total cholesterol [17], high-density lipoprotein cholesterol, low-density lipoprotein cholesterol [18], and fasting glucose [19] were estimated using available kits. Parathyroid hormone level was measured by enzyme-linked immunosorbent assay (ELISA).

Iron indices including serum iron, total iron-binding capacity (TIBC), and ferritin were determined by automated procedures carried out at the Department of Clinical Chemistry, Cairo University Hospital. TSAT was calculated by dividing the serum iron concentration by the TIBC multiplied by 100, which reflect the changes in the body iron stores. Serum high-sensitivity C-reactive protein (hsCRP) was measured by ELISA using Kit (DiaMed EuroGen, Turnhout, Belgium). The concentrations of serum interleukin 6 (IL-6) were assayed with ELISA Kits (Quantikine HS; R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. Serum hepcidin was measured using the commercially available human hepcidin ELISA Kit

(Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

Data were statistically described in terms of mean ± SD, median and range, or frequencies (number of cases) and percentages when appropriate. Comparisons between the maintenance hemodialysis patients and controls were assessed with Student's unpaired t-test, the Mann–Whitney U-test, Wilcoxon signed-rank test, or the χ^2-test, as appropriate. Correlation between various variables was made using the bivariate Pearson moment correlation equation. Univariate and multivariate regression analysis models were used to test for the independent predictors affecting hepcidin serum levels. P values less than 0.05 was considered statistically significant. All statistical calculations were performed using computer programs statistical package for the social science (SPSS, version 16; SPSS Inc., Chicago, Illinois, USA) for Microsoft Windows.

Results

The study was conducted in 40 maintenance hemodialysis patients [20 men, 20 women; age (mean ± SD) 45.3 ± 13.17 years] and in 20 age-matched healthy control individuals [11 men, nine women; age (mean ± SD) 40.55 ± 8.92 years]. There was no significant difference in age, sex, or BMI between the maintenance hemodialysis patients and healthy control individuals (Table 1).

Table 2 shows the main clinical characteristics of the study population (hemodialysis patients) just before hemodialysis session. Causes of renal failure included chronic glomerulonephritis ($n = 6$), diabetic nephropathy ($n = 8$), hypertensive nephrosclerosis ($n = 10$), polycystic kidney disease ($n = 2$), renal stone ($n = 7$), other causes ($n = 5$), or unknown ($n = 2$).

The mean plasma levels of hepcidin-25, IL-6, and hsCRP levels were significantly higher in hemodialysis patients compared with healthy controls (286.02 ± 101.17 vs. 97.05 ± 52.16, $P < 0.001$; 7.88 ± 3.65 vs. 0.44 ± 0.21, $P < 0.000$; and 5.82 ± 3.27 vs. 1.20 ± 0.92, $P < 0.000$, respectively) (Table 3 and Fig. 1).

To clarify the relationship of plasma hepcidin to the demographic, clinical, and biochemical characteristics among the hemodialysis patients, we subdivided the hemodialysis patients according to the median of hepcidin levels (calculated as 301 ng/ml) into two subgroups: subgroup 1, with hepcidin less than 301 ng/ml (lower hepcidin level) and subgroup 2, with hepcidin more than 301 ng/ml (higher hepcidin

level). Table 4 shows the main clinical characteristics of the two subgroups where the duration of dialysis, TSAT, ferritin, hepcidin, hsCRP, IL-6, and spKt/V were significantly higher, whereas hemoglobin, TIBC, UF volume, weekly epoitin-α dose, and weekly iron dose were significantly lower in subgroup 2 than in subgroup 1 (Table 4 and Figs 2 and 3).

Univariate linear regression analyses of the association of clinical and laboratory characteristics of the maintenance hemodialysis patients with serum hepcidin levels are shown in Table 5. There were significant positive correlations between serum levels of hepcidin and BMI ($r = 0.830$, $P < 0.000$), duration of dialysis ($r = 0.795$, $P < 0.000$), UF volume ($r = 0.955$, $P < 0.000$), spKt/V ($r = 0.615$, $P < 0.000$), serum iron ($r = 0.313$, $P < 0.025$), TSAT ($r = 0.710$, $P < 0.000$), ferritin ($r = 0.653$, $P < 0.000$), hsCRP ($r = 0.910$, $P < 0.000$), and IL-6 ($r = 0.867$, $P < 0.000$), and inverse correlations between serum levels of hepcidin and

Table 1 Comparison between demographic characteristics of hemodialysis patients and controls

	Hemodialysis patients	Control	P-value*
Age (years)	45.3 ± 13.17	40.55 ± 8.92	0.116
Sex (male/female) (% males)	50	55	0.76
BMI (kg/m²)	22.2150 ± 4.26912	24.6500 ± 4.59147	0.55

Data are expressed as mean ± SD; *P-value is significant if <0.05.

Table 2 Clinical characteristics of study population

Parameters	Hemodialysis patients
Duration of HDx (years)	7.60 ± 4.098
>10 years [n (%)]	8 (20)
Primary renal disease [n (%)]	
Chronic GN	6 (15)
Diabetic Nephropathy	8 (20)
Hypertensive Nephrosclerosis	10 (25)
PCKD	2 (5)
Renal Stones	7 (17.5)
Others	5 (12.5)
Unknown	2 (5)
UF volume (l/session)	2.99 ± 1.071
spKt/V (urea)	1.47 ± 0.299
Patients with spKt/V<1.2 [n (%)]	11 (27.5)
Epoitin-α	
Patients treated [n (%)]	All (100)
Dose (U/week)	4775 ± 1358.49
Iron	
Patients treated [n (%)]	All (100)
Dose (mg/week)	143.75 ± 82.57

Data are expressed as mean ± SD; EPO, erythropoietin; GN, glomerulonephritis; HDx, hemodialysis; PCKD, polycystic kidney disease; spKt/V, single pool urea clearance index (used to represent weekly dialysis dose, where K is the clearance of urea by the dialyser, t is the dialysis time, and V is the volume of distribution of urea); UF; ultrafiltration.

hemoglobin ($r = -0.783$, $P < 0.000$), TIBC ($r = -0.477$, $P < 0.001$), weekly epoitin-α dose ($r = -0.922$, $P < 0.000$), and weekly iron dose ($r = -0.793$, $P < 0.000$). No correlations were observed between serum hepcidin and the various other clinical and laboratory parameters measured (Table 5 and Figs 4–8).

Multiple linear regression analysis showed duration of dialysis, spKt/V, serum ferritin, hsCRP, IL-6, TIBC, weekly epoitin-α dose, and weekly iron doses to be independently associated with serum hepcidin levels (Table 6).

Discussion

Anemia in maintenance hemodialysis patients is a multifactorial condition and its clinical management

Table 3 Comparison between laboratory characteristics of hemodialysis patients and controls

	Hemodialysis patients	Controls	*P*-value
Hb	10.25 ± 0.87	13.06 ± 0.88	0.000*
Albumin	4.13 ± 0.33	4.54 ± 0.35	0.015*
Ca	8.20 ± 0.85	9.82 ± 0.45	0.000*
Phosphorus	5.24 ± 2.18	3.67 ± 0.60	0.026*
Ca×P product	42.64 ± 16.24	36.0 ± 6.11	0.084
iPTH	315.78 ± 287.74	36.50 ± 17.42	0.000*
TGs	151.25 ± 85.56	131.15 ± 69.48	2.06
Cholesterol	179.50 ± 41.02	136.45 ± 12.13	0.000*
LDL	105.70 ± 35.15	96.05 ± 18.37	0.281
HDL	38.4 ± 8.39	42.95 ± 9.93	0.130
Serum iron	70.30 ± 24.26	101.65 ± 36.15	0.000*
TIBC	263.42 ± 113.51	291.10 ± 60.20	0.984
TSAT	29.13 ± 12.28	34.38 ± 10.59	0.005*
Ferritin	484.89 ± 443.83	140.80 ± 79.84	0.013*
hsCRP	5.82 ± 3.27	1.20 ± 0.92	0.000*
IL-6	7.88 ± 3.65	0.44 ± 0.21	0.000*
Hepcidin-25	286.02 ± 101.17	97.05 ± 52.16	0.000*

Data are expressed as mean ± SD; Ca, calcium; Hb, hemoglobin; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; iPTH, intact parathormone; LDL, low-density lipoprotein; P, phosphorus; TGs, triglycerides; TIBC, total iron-binding capacity; TSAT, transferrin saturation; *P*-value is significant if <0.05.

Figure 1
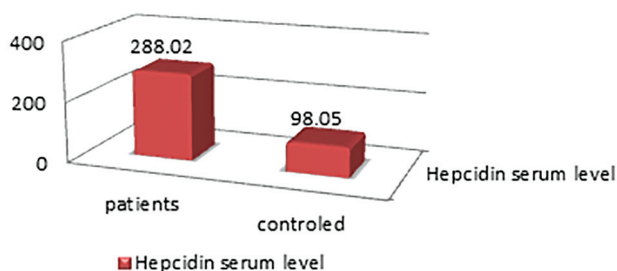
Comparison between plasma levels of hepcidin in hemodialysis patients and healthy controls.

remains challenging. The interactions between iron metabolism, EPO deficiency, and chronic inflammation are difficult to dissect and new markers are urgently needed to optimize treatment approaches [3].

Hepcidin was originally described as an antimicrobial peptide produced by the liver [5], but its main biological role is in the regulation of body iron homeostasis through interactions with ferroportin [6,7]. Hepcidin might, therefore, potentially be used as a clinical marker to optimize treatment approaches in several systemic iron disorders. It suppresses iron absorption from the intestine and stimulates iron retention in cells expressing ferroportin, notably macrophages and hepatocytes [11].

In agreement with previous data [3,20,21], the present study demonstrated that serum hepcidin levels were higher in maintenance hemodialysis patients than in healthy control individuals. The observation that there was a significant and independent correlation between hepcidin and ferritin levels suggests that it plays a major role in regulating iron homeostasis in this patient group. Our results also agreed with the results of Babitt *et al.* [8] who reported that hepcidin levels are likely to be higher in CKD patients due to limited hepcidin excretion, tissue iron overload, and inflammation.

In univariate linear regression analysis, plasma hepcidin was correlated positively with serum iron ($r = 0.313$, $P < 0.025$), TSAT ($r = 0.710$, $P < 0.000$), and ferritin ($r = 0.653$, $P < 0.000$). Multiple linear regression analysis revealed serum ferritin and TIBC as significant independent predictors for increased and decreased serum hepcidin-25 levels, respectively.

This likely reflects the known regulation of hepcidin by iron stores, which was previously demonstrated
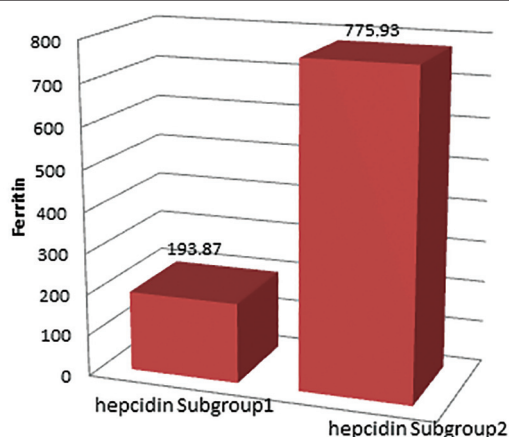
Figure 2
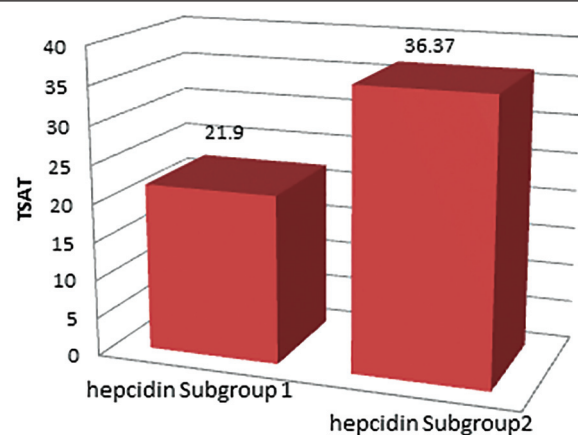
Comparison of transferrin among hemodialysis hepcidin subgroups (subgroup 1 and subgroup 2). TSAT, transferrin saturation.

Table 4 Demographic, clinical, and biochemical characteristics in all patients and in hepcidin subgroup 1 and subgroup 2

	All (n = 40)	Subgroup 1 (n = 20)	Subgroup 2 (n = 20)	P-value
Age (years)	45.3 ± 13.17	45.55 ± 13.51	45.05 ± 13.17	0.906
HDx duration (years)	7.60 ± 4.09	5.10 ± 3.21*	10.10 ± 3.31*	0.000*
>10 years [n (%)]		1 (5)*	7 (35)*	
Hemoglobin (g/dl)	10.25 ± 0.87	10.75 ± 0.91*	9.75 ± 0.44*	0.000*
Patients within target Hb (10.5>g/dl) [n (%)]		17 (85)*	2 (10)*	
Serum iron (µg/dl)	70.30 ± 24.26	64.65 ± 26.91*	75.95 ± 20.42*	0.14
TIBC (µg/dl)	263.42 ± 113.51	315.35 ± 138.05*	211.50 ± 41.40*	0.003*
TSAT (%)	29.13 ± 12.28	21.9 ± 10.39*	36.37 ± 9.55*	0.000*
Patients within target TSAT (20–40%) [n (%)]		7 (35)*	14 (70)*	
Patients with TSAT>40% [n (%)]		1 (5)*	6 (30)*	
Ferritin (ng/ml)	484.89 ± 443.83	193.87 ± 114.30*	775.92 ± 461.49*	0.000*
Patients within target ferritin (100–800 ng/ml) [n (%)]		16 (80)*	11 (55)*	
Patients with ferritin>800 ng/ml [n (%)]		–	9 (45)*	
Hepcidin (ng/ml)	286.02 ± 101.17	200.35 ± 57.54*	371.69 ± 48.98*	0.000*
hsCRP (mg/l)	5.82 ± 3.27	3.45 ± 2.34*	8.19 ± 2.15*	0.000*
Patients with CRP>5 mg/l [n (%)]		4 (20)*	18 (90)*	
IL-6 (pg/ml)	7.88 ± 3.65	5.29 ± 2.41*	10.46 ± 2.74*	0.000*
Albumin (g/dl)	4.13 ± 0.33	*4.33 ± 0.31	3.93 ± 0.20	0.000*
Patients with albumin<4 g/dl [n (%)]		3 (15)*%	8 (40)*	
Ca (mg/dl)	8.20 ± 0.85	8.92 ± 0.37*	7.48 ± 0.49*	0.000*
Phosphorus (mg/dl)	5.24 ± 2.18	4.87 ± 1.48*	5.61 ± 2.69*	0.28
iPTH (pg/ml)	315.78 ± 287.74	306.1 ± 241.58*	325.45 ± 333.75*	0.83
UF volume (l/session)	2.99 ± 1.07	3.88 ± 0.55*	2.1 ± 0.62*	0.000*
spKt/V	1.47 ± 0.29	1.33 ± 0.32*	1.61 ± 0.2*	0.002*
Patients with spKt/V<1.2 [n (%)]		11 (55)*	0 (0)*	
Epoitin-α				
Patients treated [n (%)]		All (100)*	All (100)	
Dose (U/week)	4775 ± 1358.49	5800 ± 1005.24*	3750 ± 752.19*	0.000*
Iron				
Patients treated [n (%)]		All (100)*	All (100)*	
Dose (mg/week)	143.75 ± 82.57	195 ± 64.68*	92.5 ± 65.44*	0.000*

Data are expressed as mean ± SD; Ca, calcium; Hb, hemoglobin; HDx, hemodialysis; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; iPTH, intact parathormone; spKt/V, single pool urea clearance index (used to represent weekly dialysis dose, where K is the clearance of urea by the dialyser, t is the dialysis time, and V is the volume of distribution of urea); TIBC; total iron-binding capacity; TSAT, transferrin saturation; UF; ultrafiltration; *P-value is significant if <0.05.

Figure 3

Comparison of ferritin among hemodialysis hepcidin subgroups (subgroup 1 and subgroup 2).

Figure 4
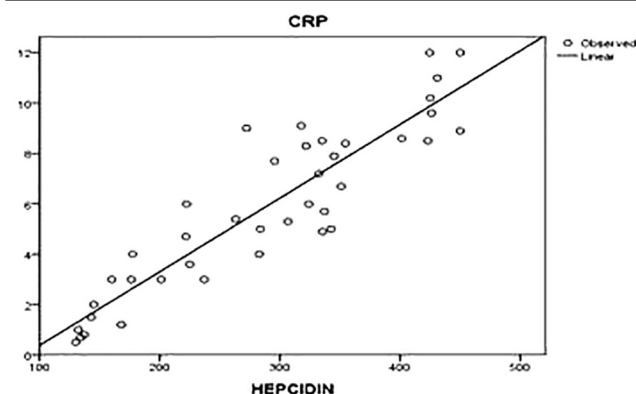
Correlation between plasma hepcidin and high-sensitivity C-reactive protein (hsCRP) in hemodialysis patients.

in populations without CKD [22]. Previous studies that used mass spectrometry to measure hepcidin also demonstrated a correlation between ferritin and hepcidin in HD patients [23,24]. In contrast, Ashby *et al.* [25], using a radioimmunoassay, did not observe this correlation in HD patients. However, a significant relationship between serum ferritin and hepcidin levels

Table 5 Correlation of plasma levels of hepcidin with demographic, clinical, and biochemical parameters in the patients

Parameters	R	P
Age (years)	0.146	0.370
Sex (male/female)	0.07	0.93
BMI (kg/m²)	0.830	0.000
Duration of dialysis (years)	0.795	0.000
Ultrafiltration volume (l)	0.955	0.000
spKt/V	0.615	0.000
Dose of epoitin-α/week	−0.922	0.000
Dose of iron	−0.793	0.000
Albumin (mg/dl)	0.826	0.000
Hb	−0.783	0.000
Serum iron	0.313	0.025
TIBC	−0.477	0.001
TSAT	0.710	0.000
Ferritin	0.653	0.000
hsCRP	0.910	0.000
IL-6	0.867	0.000
Ca	0.982	0.000
Phosphorus	0.136	0.202
iPTH	0.57	0.364

Ca, calcium; Hb, hemoglobin; hsCRP, high-sensitivity C-reactive protein; iPTH, intact parathormone; IL-6, interleukin 6; spKt/V, single pool urea clearance index (used to represent weekly dialysis dose, where K is the clearance of urea by the dialyser, t is the dialysis time, and V is the volume of distribution of urea); TIBC, total iron-binding capacity; TSAT, transferrin saturation.

Table 6 Multiple linear regression model showing the clinical and laboratory parameters with a significant independent association with serum hepcidin level in hemodialysis patients

Independent variables	B coefficient	Statistical significance (P)
Duration of dialysis (years)	0.142	0.015
spKt/V	8.6	0.044
Weekly dose of epoitin-α	−0.29	0.001
Weekly dose of iron	−2.4	0.027
TIBC	−0.213	0.032
Ferritin	0.736	0.0001
hsCRP	0.735	0.008
IL-6	1.7	0.001

hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; spKt/V, single pool urea clearance index (used to represent weekly dialysis dose, where K is the clearance of urea by the dialyser, t is the dialysis time, and V is the volume of distribution of urea); TIBC, total iron-binding capacity; *P-value is significant if <0.05

is a consistent finding. Serum ferritin is a marker of iron stores in the liver and reticulo-endothelial system (RES) as well as being an acute phase protein. Fujita *et al.* [26] demonstrated that the serum ferritin level had a strong positive correlation with the hepatic level of hepcidin mRNA expression. However, it cannot be ruled out that hepcidin primarily regulates the liver iron content, which would in turn regulate serum ferritin levels by hepatic iron content, because hepatocytes and Kupffer cells also express ferroportin [27]. Further

clarification of the relationship between hepcidin regulation and iron storage is needed.

In in-vitro studies, it has been observed that the transferrin concentration regulates hepcidin mRNA expression through a hemojuvelin/bone morphogenetic protein (BMP)-2/4-dependent pathway [28], which is thought to be the main signaling pathway for iron store-derived and erythropoiesis-derived regulation [29]. In the present study, serum iron and TSAT were not significant predictors of hepcidin levels in multivariate analysis.

Patients undergoing continuous dialysis are in a chronic inflammatory state. In our study, all inflammation markers including hsCRP and IL-6 were found to be higher in hemodialysis patient compared with healthy controls. The causes of highly prevalent state of inflammation in HD patients are multiple, including decreased renal function, volume overload, comorbidity, and intercurrent clinical events [30]. The effects of inflammation on the synthesis of hepcidin are well understood [9,22] and are mediated, at least in part, by IL-6 through induction and binding of signal transducer and activator of transcription 3 (STAT3) to the hepcidin gene promoter [31]. There was a significant positive correlation between serum IL-6 and serum hepcidin levels in the present study, which would seem to support this proposed mechanism. In addition, there was a significant positive correlation between serum hsCRP and serum hepcidin levels. Our findings are supported by Costa *et al.* [32] who found that the high levels of hepcidin found in HD patients could be related to an underlying chronic inflammation. Moreover, correlations between hepcidin and inflammatory markers (CRP and IL-6) in the HD group are similar to observational data derived from the nonuremic population [9,22]. It was Zaritsky *et al.* [33] who found that the correlation between hsCRP and hepcidin provides further evidence in support of the relationship between both variables through all stages of CKD. Van Der Weerd *et al.* [34] showed a strong association between hepcidin-25 and hsCRP but not with IL-6 in chronic HD patients.

Our findings are in disagreement with the findings of Ashby *et al.* [25] who found no significant correlation between serum hepcidin levels and inflammatory markers (hsCRP and IL-6) in adults with CKD and hemodialysis patients. In addition, Kato *et al.* [23] found no association between hsCRP and hepcidin in hemodialysis patients, but this may be because of the different assay method.
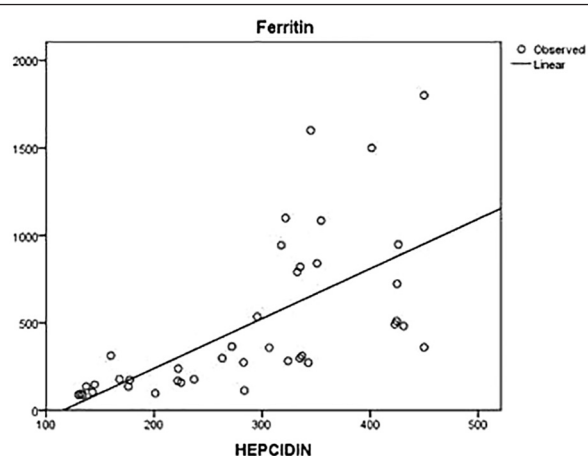
It is now recognized that uremia, as a chronic inflammatory state, upregulates hepcidin, and in particular, dialysis patients have much higher serum

Figure 5
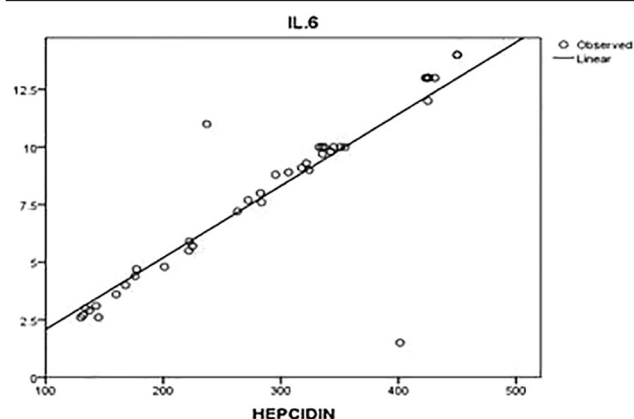
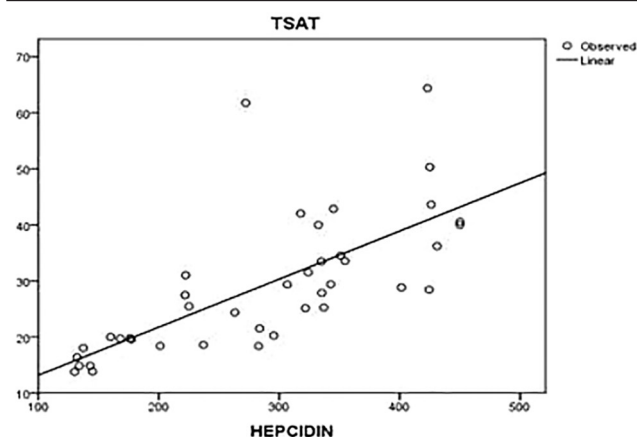Correlation between plasma hepcidin and interleukin 6 (IL-6) in hemodialysis patients.

Figure 6

Correlation between plasma hepcidin and transferrin saturation (TSAT) in hemodialysis patients.

Figure 7

Correlation between plasma hepcidin and ferritin in hemodialysis patients and controls.

Figure 8

Correlation between plasma hepcidin and hemoglobin (Hb) in hemodialysis patients.

hepcidin levels than healthy individuals [35]. It is currently believed that this has a role in the pathogenesis of anemia in CKD by limiting iron availability to the bone marrow. At a molecular level, hepcidin binds to the main iron exporter protein ferroportin, which controls iron efflux from duodenal enterocytes, hepatocytes, and macrophages [9]. The regulation of hepcidin is complex, but one of the major stimuli to its production is IL-6, produced as part of the inflammatory response. Other molecules, such as hemojuvelin and BMP-6, also have a role [36].
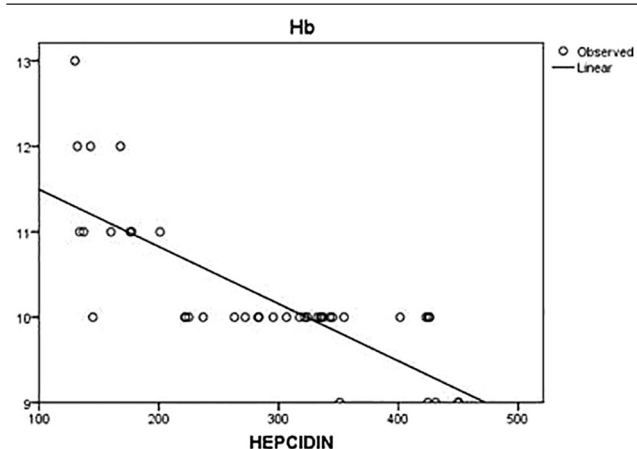
As with other inflammatory anemias, it has been hypothesized that antagonizing hepcidin may ameliorate the anemic state, and there is laboratory evidence to support this assumption. A group of scientists generated a monoclonal antibody against hepcidin and have shown that this improves anemia in an inflammatory mouse model [37]. An RNA-based antagonist of hepcidin also has been created.

It consists of a 44-nucleotide l-RNA oligonucleotide produced using so-called Spiegelmers technology (RNA molecules in which the ribose component is levorotatory, or the mirror image of the natural right-handed sugar moiety). The Spiegelmer is linked to a 40-kDa pegylation chain (NOX-H94), which has been shown to ameliorate anemia of inflammation in cynomolgus monkeys [38]. Rather than antagonizing the hepcidin molecule *per se*, another strategy could be to inhibit the production of hepcidin. This could be achieved by using antisense oligonucleotides or silencing messenger RNA transcribed from the hepcidin gene hepcidin antimicrobial peptide (HAMP). None of the strategies to suppress hepcidin production or antagonize this peptide have been subjected to clinical trials. A theoretical concern could be that inhibition of hepcidin might exacerbate the risk for infections, given its endogenous antimicrobial properties. However, there are counterarguments to this suggestion, and it

may be possible to suppress hepcidin to 'safe' levels without obliterating hepcidin activity completely [39].

In the present study, we found that there was a significant negative correlation between serum hepcidin levels and weekly iron and EPO doses. The finding that EPO treatment decreases serum hepcidin levels in patients with chronic renal failure (CRF) is in accordance with the fact that increased erythropoiesis leads to suppression of hepcidin [29]. Costa *et al.* [32] reported that EPO downregulates liver hepcidin expression, acting, therefore, as a hepcidin-inhibitory hormone. The pathways through which EPO treatment modulates hepcidin levels are largely unknown [32]. Pinto *et al.* [40] suggested that it concerns a direct effect, mediated by a decrease in CEBPA binding to the hepcidin promoter after EPO supplementation. Alternatively, EPO administration may indirectly lead to suppression of hepcidin by increased levels of growth differentiation factor-15, which is secreted by erythroblasts [41], or by the molecule named twisted gastrulation (TWSG1). TWSG1 is expressed during erythropoiesis and acts by inhibiting BMP-induced expression of hepcidin [42]. Although we studied a relatively small and heterogeneous patient population, our results with respect to the hepcidin decrease in response to EPO are unambiguous. It seems that the signaling pathway that connects EPO with hepcidin is not markedly influenced by systemic disturbances seen in patients with kidney failure.

In our study, we found a positive correlation between spKt/V and serum hepcidin level. This unexpected finding was in contradiction with the findings of Zaritsky *et al.* [43] who reported that hepcidin could be cleared efficiently by hemodialysis as it is a very small molecule. This finding was consistent with other studies [20,25] that showed no reduction following a standard dialysis session. The cause of this variability remains unclear but might be attributable to differences in the membrane of the dialyser, residual renal function, or induction of hepcidin by the hemodialysis procedure [44]. Future studies will need to address the kinetics of hepcidin clearance during dialysis. Formal kinetic modeling of hepcidin clearance will allow for estimation of the generation rate and volume of distribution of hepcidin and would be valuable for predicting hepcidin removal through various dialysis regimens.

Conclusion

Our analyses suggest that serum hepcidin levels were associated with iron status, microinflammation, anemia therapy, and parameters of dialysis efficiency in maintenance hemodialysis patients. Availability of reliable, harmonized, and preferably automated assays (e.g. competitive ELISA) should become available for routine measurement of hepcidin before hepcidin can be used in clinical practice. The key role of hepcidin in iron homeostasis and its disorders suggests that its assay could prove useful for the diagnosis, monitoring, and prognosis of iron disorders, and therefore potentially be used as a clinical marker to optimize treatment approaches. However, large and well-designed studies exploiting harmonized assays are required to firmly establish the position of Hepcidin in diagnostic medicine. Finally, hepcidin-targeted therapies may improve treatment options for patients suffering from iron disorders.

References

1 Eleftheriadis T, Liakopoulos V, Antoniadi G, Kartsios C, Stefanidis I. The role of hepcidin in iron homeostasis and anemia in hemodialysis patients. Semin Dial 2009; 22:70–77.

2 KDOQI, National Kidney Foundation. KDOQI clinical practice guidelines and clinical practice recommendations for anaemia in chronic kidney disease. Am J Kidney Dis 2006; 47:S11–S145.

3 Weiss G, Theurl I, Eder S, et al. Serum hepcidin concentration in chronic haemodialysis patients: associations and effects of dialysis, iron and erythropoietin therapy. Eur J Clin Invest 2009; 39:883–890.

4 Verga Falzacappa MV, Muckenthaler MU. Hepcidin: iron hormone and anti-microbial peptide. Gene 2005; 364:37–44.

5 Park CH, Valore EU, Waring AJ, et al. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001; 276:7806–7810.

6 Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anaemia, hypoxia, and inflammation. J Clin Invest 2002; 110:1037–1044.

7 Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. Annu Rev Nutr 2006; 26:323–342.

8 Babitt JL, Lin HY. Molecular mechanisms of hepcidin regulation: implications for the anaemia of CKD. Am J Kidney Dis 2010; 55:726–741.

9 Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004; 306:2090–2093.

10 Ganz T. Hepcidin and its role in regulating systemic iron metabolism. Hematology Am Soc Hematol Educ Program 2006; 507:29–35.

11 Cui YJ, Wu QY, Zhou YQ. Iron-refractory iron deficiency anemia: new molecular mechanisms. Kidney Int 2009; 76:1137–1141.

12 Daugirdas JT. Second generation logarithmic estimates of single-pool variable volume Kt/V: an analysis of error. J Am Soc Nephrol 1993; 4:1205–1213.

13 Rodkey FL. Binding of bromocresol green by human serum albumin. Arch Biochem Biophys 1964; 108:510–513.

14 Kessler G, Wolfman M. An automated procedure for the simultaneous determination of calcium and phosphorus. Clin Chem 1965; 45:290–296.

15 Goodwin JF. Quantification of serum inorganic phosphorus, phosphatase, and urinary phosphate without preliminary treatment. Clin Chem 1970; 16:776–780.

16 Wahlefeld AW. Triglyceride determination after enzymatic hydrolysis. In: HU Berger, editor. *Methods of enzymatic analysis.* 2nd English ed. (translated from 3rd German ed.). NY and London: Verlag Chemie Weinheim and Academic Press Inc.; 1974. 4:1813–1835.

17 Flegg HM. An investigation for the determination of serum cholesterol by an enzymatic method. Ann Clin Biochem 1973; 10:79–84.

18 Friedwald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifugation. Clin Chem 1972; 18:499–502.

19 Trinder L. Determination of blood glucose using an oxidase–peroxidase system with a non-carcinogenic chromagen. Ann Clin Biochem 1969; 22:158–161

20 Xu Y, Ding XQ, Zou JZ, et al. Serum hepcidin in haemodialysis patients: associations with iron status and microinflammation. J Int Med Res 2011; 39:19611967.

21 Tessitore N, Girelli D, Campostrini N, et al. Hepcidin is not useful as a biomarker for iron needs in haemodialysis patients on maintenance erythropoiesis-stimulating agents. Nephrol Dial Transplant 2010; 25:3996–4002.

22 Nemeth E, Valore EV, Territo M, et al. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003; 101:2461–2463.

23 Kato A, Tsuji T, Luo J, et al. Association of prohepcidin and hepcidin-25 with erythropoietin response and ferritin in hemodialysis patients. Am J Nephrol 2008; 28:115–121.

24 Tomosugi N, Kawabata H, Wakatabe R, et al. Detection of serum hepcidin in renal failure and inflammation by using ProteinChip system. Blood 2006; 108:1381–1387.

25 Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009; 75:976–981.

26 Fujita N, Sugimoto R, Takeo M, Urawa N, Mifuji R, Tanaka H, et al.; Hepcidin expression in the liver : relatively low level in patients with chronic hepatitis C. Mol Med. 2007 Jan-Feb; 13:97–104.

27 Kumar S, Raftery M, Yaqoob M, et al. Anti-inflammatory effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors (statins) in peritoneal dialysis patients. Perit Dial Int 2007; 27:283287.

28 Liu XB, Nguyen NB, Marquess KD, et al. Regulation of hepcidin and ferroportin expression by lipopolysaccharide in splenic macrophage. Blood Cells Mol Dis 2005; 35:47–56.

29 Kearney SL, E Nemeth, EJ Neufeld, et al. Urinary hepcidin in congenital chronic anemias. Pediatr Blood Cancer 2007; 48:57–63.

30 Stenvinkel P, Ketteler M, Johnson RJ, et al. IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia — the good, the bad, and the ugly. Kidney Int 2005; 67:1216–1233.

31 Wrighting DM, Andrews NC. Interleukin-6 induces hepcidin expression through STAT3. Blood 2006; 108:3204–3209.

32 Costa E, Pereira BJG, Rocha-Pereira P, et al. Role of prohepcidin, inflammatory markers and iron status in resistance to rhEPO therapy in hemodialysis patients. Am J Nephrol 2008; 28:677–683.

33 Zaritsky J, Young B, Wang HJ, et al. Hepcidin: a potential novel biomarker for iron status in chronic kidney disease. Clin J Am Soc Nephrol 2009; 4:1051–1056.

34 Van der Weerd NC, Grooteman MPC, Bots ML, et al. Hepcidin-25 in chronic hemodialysis patients is related to residual kidney function and not to treatment with erythropoiesis stimulating agents. PLoS One 2012; 7:e39783.

35 Macdougall IC, Malyszko J, Hider RC, Bansal SS. Current status of the measurement of blood hepcidin levels in chronic kidney disease. Clin J Am Soc Nephrol. 2010; 5:1681–1689.

36 Kemna EH, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. Haematologica 2008; 93:90–97.

37 Sasu BJ, Cooke KS, Arvedson TL, et al. Antihepcidin antibody treatment modulates iron metabolism and is effective in a mouse model of inflammation-induced anemia. Blood 2010; 115:3616–3624.

38 Noxxon Pharma AG. 2011. Pipeline. NOX-H94, 44-nucleotide l-RNA oligonucleotide linked to 40 kDa PEG. Available at: *http://www.noxxon. com/index.php?option_com_content&view_article&id_88&Itemid_100*. [Accessed 29 May 2011]

39 Macdougall IC. New anemia therapies: translating novel strategies from bench to bedside. Am J Kidney Dis 2012; 59:444–451.

40 Pinto JP, Ribeiro S, Pontes H, et al. Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. Blood 2008; 111:5727–5733.

41 Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med 2007; 13:1096–1101.

42 Tanno T, Porayette P, Sripichai O, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood 2009; 114:181–186.

43 Zaritsky J, Young B, Gales B, et al. Reduction of serum hepcidin by haemodialysis in pediatric and adult patients. Clin J Am Soc Nephrol 2010; 5:1010–1014.

44 Swinkels DW, Wetzels JFM. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? Nephrol Dial Transplant 2008; 23:2450–2453.