Study of hepcidin 25 as a novel iron biomarker in hemodialysis chronic kidney disease patients at Urology and Nephrology Minia University Hospital

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ABSTRACT

BACKGROUND: Hepcidin levels rise as a result of the chronic inflammation found in CKD. Hepcidin decreases the amount of iron released from iron stores and absorbed in gut, which contributes to functional iron deficiency.

OBJECTIVES: We evaluated the hepcidin role compared to inflammation and parameters of iron in chronic hemodialysis patients.

PATIENTS AND METHODS: 86 participants were included and divided into 2 groups, the control group (21 healthy persons), the hemodialysis CKD group (65 patients). The patients received regular erythropoietin therapy before the study. All of them were subjected to thorough history taking and complete clinical examination. CBC, viral hepatitis markers, kidney function tests, serum levels of hepcidin-25, tumor necrosis factor-α(TNF-α), interleukin-6 (IL-6), high sensitive C-reactive protein (hs-CRP), Transferrin saturation (TSAT), iron, ferritin, and total iron binding capacity (TIBC) were assessed for all subjects.

RESULTS: The hemodialysis group had significantly higher serum levels of IL-6, TNF-α, and hs-CRP than the control group. Serum iron and TSAT levels were significantly greater in HD-CKD patients compared to controls, but Hb and TIBC values were significantly lower. When compared to the controls, serum hepcidin-25 was considerably higher in the HD patients. In the patient group, hepcidin showed a positive correlation with serum iron, TSAT, ferritin, TNF-α, hs-CRP, IL-6, blood urea and creatinine and a negative correlation with Hb level and TIBC. Stepwise regression analysis revealed that hepcidin was significantly predicted by Hb, ferritin, TSAT, TNF-α, IL-6 and hs-CRP. Hepcidin and ferritin were significant predictor of functional iron deficiency anemia (AUC=.791, AUC=.724, respectively). Hepcidin levels can be considerably predicted by serum levels of ferritin, iron, IL-6, and hs-CRP. When compared high hepcidin patients to low hepcidin patients, hepcidin, iron, ferritin, IL-6 and hs-CRP were significantly increased while Hb was noticeably decreased.

CONCLUSIONS: Serum hepcidin-25 is a reliable biomarker for iron status in HD-CKD patients. Additionally, determining hepcidin in conjunction with markers linked to iron metabolism improves the ability to identify patients who are iron deficient.

Key words: Serum hepcidin-25; CKD; Hemodialysis

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INTRODUCTION

Hepcidin is a 25 amino acid peptide and its bioactive form is hepcidin 25.[1] While the liver is the primary site of production of systemic hepcidin, it is also present in the kidney tubules, heart, lungs, as well as in monocytes, macrophages, and neutrophils.[2] Inflammation and increased iron body availability both promote hepatic hepcidin synthesis. Hypoxia, elevated erythropoiesis, and low circulating iron levels all hinder it.[3] Iron from diet is absorbed in upper jejunum and duodenum. The efflux of cellular iron into systemic circulation is mediated by the iron transporter ferroportin (FPN) at the basolateral location of enterocytes. Hepatocytes, macrophages, and enterocytes are a few examples of cells that export iron and have membranes where hepcidin binds to FPN. Iron transport to plasma and the supply of iron for erythropoiesis are both decreased by ferroportin's endocytosis and lysosomal breakdown as a result of hepcidin binding to it.[4] Hepcidin is more concentrated in CKD due to the presence of inflammation, decreased kidney hepcidin filtration, and decreased erythropoietin levels.[5]

Chronic inflammation is now recognised as a distinguishing characteristic of chronic renal disease. This chronic inflammation can be caused by many factors, including increased production of

proinflammatory cytokines, acidosis, oxidative stress, recurrent and persistent infections, intestinal dysbiosis and altered adipose tissue metabolism. Hemodialysis-related systemic inflammation increases the risk of mortality and triggers various complications such as depression, osteoporosis, premature vascular disease, muscle atrophy, and vascular calcification.[6]

Iron deficiency is widespread among CKD patients who are dialysis and non-dialysis dependant because of the concomitant reduction in iron absorption and increase in losses of iron. Absolute iron insufficiency and functional iron shortage are the 2 types of iron deficiency that are recognised. Severely diminished or nonexistent iron stores in liver, bone marrow and spleen are indicators of absolute iron insufficiency. Normal or increasing total iron reserves in body that cannot be incorporated into erythroid precursors for erythropoiesis are considered to have functional iron deficiency. Increased levels of hepcidin, which hinder the recruitment of iron reserves from hepatocytes and reticuloendothelial for erythropoiesis, are the primary cause of functional iron shortage.[7]

PATIENTS AND METHODS

Our cross sectional observational study was conducted at Urology and Nephrology Minia University Hospital in Minia, Egypt from March 2022 to December 2022. Each case provided written consent, which the hospital ethics committee authorised. There were 86 participants in the study. The included subjects were divided into two groups. Group A consisted of 21 healthy adult participants who served as the study's control group. Group B consisted of 65 chronic hemodialysis patients who had been chosen from the hemodialysis unit at the Urology and Nephrology University Hospital and had been receiving hemodialysis for at least six months. All HD patients were receiving regular erythropoietin treatment. In our study we excluded diabetic patients, malignant diseases, patients with chronic liver diseases, patients with autoimmune diseases, acute infections, recent burns, pregnant women, patients with history of blood transfusions 3 months before the research and patients with recent surgery. A detailed history was taken of each participant, followed by a clinical examination. Age, sex, medication history (erythropoietin and iron dosages), hemodialysis duration and frequency per week, and history of HTN were all taken into account during the history-taking process. Vital signs, specially assessment of blood pressure, body mass index (BMI), complete cardiac, abdomen, chest, and neurological evaluation were all part of the clinical examination.

Each individual had a sterile venipuncture performed under strictly aseptic settings to extract approximately 6 ml of venous blood from a peripheral vein. After an overnight fast, a sample was taken from individuals with HD-CKD at the beginning of a hemodialysis session. After an overnight fast, a peripheral vein was also used to obtain venous blood samples from healthy controls. For the purpose of determining the CBC, 1 ml was put in a tube containing ethylene diamine tetraacetic acid (EDTA). 5 ml were centrifuged for 15 minutes at about 3000 rpm after being allowed to clot for 30 minutes in a plain tube. Expressed serum was gathered and kept at -70°C for analysis. This serum was utilised to analyse routine laboratory investigations as kidney function tests, viral hepatitis markers, liver biochemistry tests, fasting and 2 hours postprandial blood glucose levels, serum iron, serum ferritin and serum TIBC. Additionally, it was used to detect particular investigations such as quantitative measurement of serum TNF-α, serum IL-6, serum hs-CRP and bioactive hepcidin using enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

Version 25 of SPSS was utilised to code, tabulate, and statistically analyse the obtained data. The mean, standard deviation (SD), median and interquartile range (IQR)were used to compute descriptive statistics for numerical data, while number and percentage were used to construct them for categorical data. When comparing the means of 2 groups in analyses of quantitative parametric variables, independent T test was used. Mann Whitney test was used to evaluate non-parametric quantitative variables for comparisons involving two groups. For comparing qualitative data between groups, the chi square test was used. Quantitative variables were correlated by using Pearson's correlation coefficient. Stepwise regression analysis was used to detect the relation between hepcidin and the laboratory parameters. Receiver operating characteristic (ROC) curve was utilised to detect predictor of anemia and predictors of hepcidin in our study.

RESULTS

Hemodialysis patients which made up of 33 males (50.8%) and 32 females (49.2%) had a median age of 47(7.5) years. HTN was found in 47 patients (72.3%) and 6 controls (28.6%) overall. HTN was statistically increased in the patients compared to the controls (P<.001). Comparing the patients to the controls revealed a statistically significant decrease in BMI (P<.001). Serum levels of the inflammatory markers, TNF-α, IL-6 and hs-CRP were found higher in the patients' group of our study compared to the healthy individuals. HD patients had a considerably higher level of serum hepcidin-25 than the controls did (P<.001) **(Table 1)**. The studied patients had significantly higher serum iron levels (P=.03) and TSAT (P=.04) than the normal controls. In comparison to the control

subjects, the serum ferritin levels were considerably higher in the patients' group (P<.001). When compared to the healthy persons, HD-CKD patients had significantly lower levels of haemoglobin (P<.001) **(Table 2)**.

Regarding correlation analysis of hepcidin in the dialysis CKD patients, levels of hepcidin-25 were positively linked with those of hs-CRP (P<.001), IL-6 (P<.001) and TNF-α (P=.01). Our study also discovered statistically positive link between hepcidin-25 and serum creatinine (P=.007) and blood urea (P=.007). As regards correlation of hepcidin with the iron profile, it was positively correlated with serum iron (P<.001), TSAT (P<.001) and serum ferritin (P<.001) despite being negatively correlated with Hb (P<.001) and TIBC (P<.001) **(Table 3)**. Stepwise regression analysis in the patients under study as shown in **(Table 4)** revealed that Hb, ferritin, TSAT and the inflammatory markers including IL-6, hs-CRP and TNF- α were significant determinants of hepcidin.

ROC curve analysis as illustrated in **Figure 1** revealed that hepcidin-25 with cut-off value >26 ng/ml was a strong predictor of functional iron deficiency anemia in our CKD patients (AUC = 0.791, sensitivity = 87.8%, specificity = 68.7%, P<.001). Ferritin level was also a significant predictor of anemia (AUC=.742, P=.007) **(Table 5)**. Serum ferritin (AUC=.852 & P<.001) and serum iron (AUC=.678 & P=.03) were strong predictors of hepcidin level. Hepcidin level was also predicted by levels of hs-CRP (AUC=.691 & P=.02) and IL-6 (AUC=.774 & P=.001) **(Table 6)**.

The patients were subsequently separated into two groups, low hepcidin and high hepcidin groups, based on the degree of hepcidin that predicted anemia. There was significant increase in levels of hepcidin (P<.001), iron (P=.03) and ferritin (P<.001) of high hepcidin group when compared to low hepcidin group. Patients with high hepcidin levels experienced lower Hb levels than those with low hepcidin levels (P<.001). High hepcidin group was found to have considerably higher levels of IL-6 and hs-CRP than low hepcidin group **(Table 7)**.

Table 1. Demographic characteristics and inflammatory markers of the groups under study

Variable	**Group A (Controls) n = 21**	**Group B (HD group) n = 65**	**P**
Age (years)	44.6(6) 52 – 38	47(7.5) 56 – 40	.06
Sex Male Female	 12 (57.1%) 9 (42.9%)	 33 (50.8%) 32 (49.2%)	 .6
HTN	6 (28.6%)	47 (72.3%)	**<.001**
Body mass index (BMI) (kg/m^2)	23.5 (1) 26.8 – 21	22.2 (1) 25.1 – 20	**<.001**
Serum creatinine (mg/dl)	.9 (.15) 1.1 - .5	5.8 (.6) 8.4 – 4	**<.001**
Blood urea (mg/dl)	27(5.5) 33 – 19	134(8) 141 – 67	**<.001**
High sensitive C-reative protein (mg/L)	6(6) 12 – 0	24 (32) 72 – 6	**<.001**
Interleukin-6(IL-6) (pg/ml)	5.6 (2.1) 10.8 – 1.5	13.4 (10.9) 42 – 4.5	**<.001**
Tumor necrosis factor-α (TNF- α) (pg/ml)	7.5 (3.5) 10.5 – 3.7	25(18) 40 – 9.8	**<.001**
Hepcidin-25 (ng/ml)	6.5 (2.7) 9 – 4.2	35(45.5) 96 – 17.5	**<.001**

Data are n (%) or median (interquartile range, maximum-minimum)

Table 2. Iron profile of the controls and hemodialysis group

Variable	**Group A (Controls) n = 21**	**Group B (HD group) n = 65**	**P**
Serum iron (µg/dl)	135 (37.5) 175 – 77	158(57.5) 340 – 100	**.03**
Total binding iron capacity (TIBC) (µg/dl)	275(32.5) 339 – 209	270(90) 325 – 135	.09
Serum ferritin (ng/ml)	89(55.5) 199 – 38	336(115) 728 – 240	**<.001**
Transferrin Saturation (TSAT) (%)	50(17.7) 81.3 - 24.8	58(48.2) 237 – 32.7	**.04**
Hb (gm/dl)	13(0.9) 14.5 - 12.5	9.2 (3.1) 13.5 – 6.5	**<.001**

Data are median (interquartile range, maximum-minimum)

Table 3. Correlation analysis of hepcidin with laboratory investigations in HD group

Variable	**HD patients**	
	r	**P**
hs CRP	.57	**<.001**
IL-6	.49	**<.001**
TNF-α	.3	**.01**
Serum iron	.64	**<.001**
TIBC	-.62	**<.001**
Ferritin	.59	**<.001**
TSAT	.65	**<.001**
Hb	- 0.57	**<.001**
Creatinine	.33	**.007**
Blood urea	.33	**.007**

Table 4. Stepwise regression analysis in HD patients for hepcidin determinants

		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	T	P
1	(Constant)	22.087	4.241		5.208	.000
	TSAT	.300	.044	.648	6.755	**.000**
2	(Constant)	-7.239-	6.749		-1.073-	.288
	TSAT	.237	.040	.511	5.980	**.000**
	Ferritin	.095	.018	.438	5.127	**.000**
3	(Constant)	42.527	13.954		3.048	.003
	TSAT	.214	.036	.461	5.928	**.000**
	Ferritin	.070	.018	.326	3.982	**.000**
	HB	-4.096-	1.034	-.319-	-3.960-	**.000**
4	(Constant)	40.056	12.282		3.261	.002
	TSAT	.166	.034	.358	4.951	**.000**
	Ferritin	.056	.016	.259	3.528	**.001**
	HB	-4.248-	.910	-.331-	-4.668-	**.000**
	CRP	.470	.108	.309	4.348	**.000**
5	(Constant)	33.417	12.053		2.773	.007
	TSAT	.141	.034	.305	4.210	**.000**
	Ferritin	.050	.015	.229	3.204	**.002**
	HB	-4.021-	.876	-.313-	-4.589-	**.000**
	CRP	.476	.104	.313	4.590	**.000**
	IL6	.577	.228	.173	2.531	**.014**
6	(Constant)	26.102	12.179		2.143	.036
	TSAT	.146	.033	.316	4.483	**.000**
	Ferritin	.050	.015	.231	3.326	**.002**
	HB	-3.966-	.851	-.309-	-4.662-	**.000**
	CRP	.414	.105	.272	3.953	**.000**
	IL6	.529	.222	.159	2.377	**.021**
	TNF	.360	.167	.135	2.155	**.035**

Table 5. ROC curve analysis of hepcidin and iron profile to predict functional iron deficiency anemia in our HD patients

Variable	**AUC**	**P**	95% Confidence Interval	
			Lowe bound	Upper bound
Hepcidin	.791	**.001**	.659	.923
Ferritin	.724	**.007**	.577	.872
iron	.588	.293	.419	.757
TSAT	.622	.144	.462	.782
TIBC	.360	.094	.196	.524

AUC: area under the curve

Table 6. ROC curve of iron parameters and inflammatory markers to predict hepcidin level

Variable	**AUC**	**P**	95% Confidence Interval	
			Lowe bound	Upper bound
Ferritin	.852	**<.001**	.752	.953
iron	.678	**.03**	.540	.816
TSAT	.626	.126	.483	.768
TIBC	.428	.378	.288	.567
TNF	.600	.224	.451	.748
CRP	.691	**.02**	.556	.827
IL6	.774	**.001**	.639	.909

AUC: area under the curve

Table 7. Comparison of laboratory variables in low and high hepcidin groups

Variable	**High hepcidin group (>26 ng/ml) n = 48**	**Low hepcidin group (<26 ng/ml) n = 17**	**P**
Hepcidin-25 (ng/ml)	40 (49.6) 96 – 26.5	23.5(4.3) 26 – 17.5	**<.001**
Serum iron (µg/dl)	170(73) 340 – 103	150(42) 195 – 100	**.03**
TIBC(µg/dl)	270(115.8) 325 – 135	266(45) 315 – 210	.37
Serum ferritin (ng/ml)	335(201) 728 – 249	266 (74.5) 356 – 240	**<.001**
TSAT (%)	58.2 (77.4) 237 – 36.1	53.6 (20.8) 92.9 – 32.7	.12
Hb (gm/dl)	8.5(1.9) 13 – 6.5	12.4(3.1) 13.5 – 9	**<.001**
hs-CRP (mg/L)	24(34.5) 72 – 6	18(15) 40 – 6	**.01**
IL-6 (pg/ml)	17 (9.8) 34.8 – 5.7	9 (5.3) 42 – 4.5	**.001**
TNF- α (pg/ml)	25(16.9) 40 – 9.8	22(17) 34 – 10.5	.22

Data are median (interquartile range, maximum-minimum)

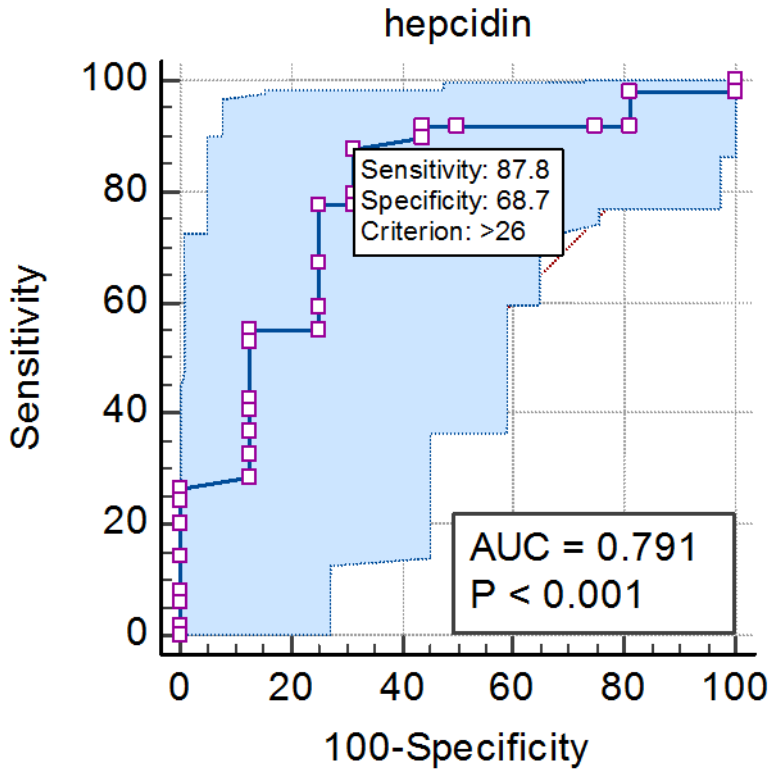

Figure 1. ROC curve of hepcidin for prediction of anemia in HD patients

DISCUSSION

Inflammation characterizes CKD and there are numerous causes of inflammatory activation in CKD patients. Despite reports of increased production, higher levels of circulating cytokines are undoubtedly caused by a reduction in renal clearance. Oxidative stress and carbonyl stress, both of which are extremely pro-inflammatory, are brought on by the uremic environment. It has been suggested that uremic toxins produce intestinal dysbiosis and enhance the translocation of gut bacteria and bacterial components into the circulation, which can lead to systemic inflammation.[8] HD-CKD patients' serum levels of hs-CRP, IL-6, and TNF-α were considerably higher than those of the control subjects. This agrees with the findings of Mohamed et al. who discovered that the levels of CRP and TNF-α were statistically higher in dialysis patients [9]. This is also in line with Zahed et al who showed that IL-6 was noticeably increased in hemodialysis individuals.[10]

Serum hepcidin-25 levels were considerably greater in the patient group. Similar findings were found in Kamal et al. study which revealed that there was a significant increase of hepcidin levels in hemodialysis and non-hemodialysis CKD patients.[11] Reduced renal clearance of hepcidin is what causes the increased serum levels of hepcidin-25. It has been discovered that a variety of inflammatory cytokines, predominantly IL-6, but also IL-1, IL-22, and interferon, favourably up-regulate the expression of hepcidin.[12] This further suggests an underlying inflammatory condition linked to progressive renal failure.[13]

Serum ferritin levels were considerably higher in all patients compared to controls. Despite having lower levels of TIBC, the patients had noticeably greater levels of TSAT and serum iron as compared to the control group. Our study also found that the HB level was significantly lower in the patients. This is consistent with the findings of the study by Reedy et al., which showed that hemodialysis patients had much higher serum iron, serum ferritin and TSAT levels compared to healthy controls, although their TIBC and haemoglobin levels were significantly lower.[14] This is also in agreement with Sarhan et al. study which revealed that the dialysis group had increased serum ferritin, serum iron and TSAT when compared to the normal controls, although their Hb level was significantly lower.[15]

We found in our study that hepcidin was positively linked with serum ferritin, serum iron and TSAT In spite of being adversely associated with TIBC and Hb. This is consistent with Sarhan et al. study which found that hepcidin had a positive relationship with serum ferritin, serum iron and TSAT and a negative correlation with Hb level.[15]

This also agrees with Ibrahim et al. findings.[16] Stepwise regression analysis of our study showed that serum iron, TSAT, and serum ferritin were strong predictors and determinants of hepcidin level. This is similar to findings of Xu et al. study which revealed that hepcidin of hemodialysis patients was positively associated with ferritin and TSAT and negatively linked with TIBC.[17] Hepcidin was found to be a strong predictor of functional iron deficiency anemia in our study which is similar to findings of Sarhan et al and Nalado et al. studies.[15, 18]

Anemia is one of the most prevalent comorbidities of CKD and is brought on by a number of reasons, including dietary deficiencies (such as folate and vitamin B12 deficiency) and a decline in endogenous erythropoietin synthesis in the kidneys. Recombinant human erythropoietin (rHuEpo) was created to treat anemia in patients with CKD, whose primary therapy involved repeated blood transfusions that led to secondary iron overload. Despite the fact that rHuEpo lowers the risks of iron overload and protects patients from becoming transfusion dependent, it still has a significant impact on anemia in CKD patients. Despite the fact that rHuEpo delivers oxygen, it also has the potential to kill cells by generating free radicals, hence systemic iron levels must be controlled.[19] Iron is recycled from senescent erythrocytes in healthy individuals and is transferred into the bone marrow via the RES where it is mixed with erythroblasts. In order to maintain normal iron homeostasis, daily loss is made up for by duodenal absorption of dietary iron. Absolute iron shortage is likely to occur in CKD patients as a result of decreased total body iron storage due to increased blood loss, particularly in dialysis-dependent CKD patients, and perhaps impaired gastrointestinal iron absorption. In addition, many CKD patients exhibit functional iron insufficiency because of reticuloendothelial cell iron blockage, which is defined as the inability to transfer enough iron to the site of erythroblast formation while having appropriate reserves.[19] Functional iron insufficiency is primarily caused by elevated hepcidin levels, which prevent recruitment of iron reserves from hepatocytes and reticuloendothelial cells for erythropoiesis.[20]

According to our study, functional anemias, which are characterised by lower haemoglobin levels in the context of elevated serum ferritin and normal or elevated serum iron levels, are the most common type of anemia seen in CKD patients. IV iron is administered combined with EPO and hemopoietic supplements to treat anemia. Patients with HD who take iron supplements respond better to EPO therapy, replenish ongoing iron losses, and maintain target haemoglobin and hematocrit levels.[14]

ROC curve of inflammatory markers and iron parameters in our data illustrated that hepcidin was predicted by serum levels of ferritin, iron, IL-7 and hs-CRP. This is because hepcidin synthesis can by stimulated by increased iron availability and inflammation.[3] Serum hepcidin and serum creatinine of patients in our research revealed a positive correlation that is similar to the study of Troutt et al.[21] It is explained by decreased renal clearance in renal failure that results in increased serum level of hepcidin.[13] We found that serum hepcidin-25 was positively correlated with serum IL-6, serum hs-CRP and serum TNF-α. Stepwise regression of our patients also found that the markers of inflammation including TNF-α, hs-CRP and IL-6 were significant determinants and strong predictors of hepcidin. This supports the findings of Xu et al. who found that hepcidin of hemodialysis patients was positively associated with IL-6.[17] This is also in line with findings of Ibrahim et al. study which discovered that hepcidin was positively linked with CRP and IL-6.[16] An underlying inflammatory condition linked to progressive renal failure explains this.[13] Numerous cytokines have been found to positively up-regulate the expression of hepcidin, most notably IL-6, but also TNF-α, hs-CRP, and interferon.[12]

There are some limitations related to our study. First, the results aren't generalizable to the total population with chronic renal disease because it was a single-center study with a tiny sample size. Second, the blood hemodialysis procedure may have an impact on the hepcidin level. Hepcidin levels were only tested once, so it's impossible to say with certainty whether they changed during the course of the study. The study does not permit a cause-and-effect relationship; only an association can be mentioned.

In conclusion, inflammation in CKD increases hepcidin levels which result in a reduction in the amount of iron that is absorbed in the gut and released from iron reserves. This helps to cause functional iron insufficiency and erythropoiesis-stimulating agents' resistance.

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