Role of Hepcidin as A Predictor for Iron Metabolism Disorders and Inflammation in Stage 4 Chronic Kidney Disease Patient with Anemia

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Abstract

Background: Anemia is more frequent in cases with chronic kidney disease compared to the general population. **Aim:** To assess the role of hepcidin as a predictor for iron metabolism disorders in stage 4 CKD cases with anemia and to evaluate the role of hepcidin as a predictor for inflammation in stage four chronic kidney disease cases with anemia. **Patients and methods:** This case-control cross-sectional observational research has been conducted on 64 cases with CKD stage 4 with anemia divided into four groups: Group (A): involved 16 cases with hemoglobin levels below 6 g/dl; Group (B): included 16 cases with hemoglobin concentrations six to nine gram per deciliters; Group (C): included 16 patients with hemoglobin levels above 9-11 g/dl; and Group (D): included 16 healthy subjects as a control group at **the** Nephrology Unit of the Internal Medicine Department in Assiut University Hospitals from April 2020 till July 2022. **Results:** Utilizing the ROC curve, it has been demonstrated that hepcidin may be utilized to discriminate among anemic groups and the control group at a cutoff level of > 6.6 ng/ml, with 100% specificity, 93.7% sensitivity, 100% PPV, and 94.07% NPV (p-value < 0.001 & AUC = 0.996). **Conclusion:** Hepcidin was a good predictor for iron metabolism disorders and inflammation in stage 4 CKD patients with anemia. Hepcidin and CRP levels were the highest among patients with Hgb levels below 6 gm/dl. Hepcidin level correlated positively with the increase of ferritin in all patients.

Key words: Hepcidin, iron metabolism disorders, CKD

Introduction

Anemia is more common in cases with chronic kidney disease in comparison with the general population. Moreover, anemia is typically more marked in cases with advanced chronic kidney disease [1]. The presence of anemia in chronic kidney disease cases elevates the possibility of ESRD, cardiovascular diseases, and mortality; thus, identifying factors correlated with anemia in this population is crucial. The primary factor leading to anemia in cases with chronic kidney disease is erythropoietin deficiency. Erythropoietin is synthesized by kidney interstitial fibroblasts and promotes RBC production in bone marrow [2]. With the advancement of chronic kidney disease, erythropoietin concentrations become inadequate relative to the severity of anemia, potentially resulting in normochromic, normocytic, and hypo-proliferative anemia. Efforts to rectify erythropoietin deficiency have involved normalizing Hgb concentrations through erythropoietin replacement; however, these attempts have eventually not enhanced clinical results. Altered iron metabolism was proposed as an additional factor that causes anemia in cases with chronic kidney disease [3]. Traditionally, transferrin saturation (TSAT) and ferritin were utilized as serum iron indicators. Transferrin saturation, the ratio of serum iron to total iron-binding capacity, acts as an indicator of accessible serum iron. Serum

ferritin levels, an intracellular iron storage protein, serve as an indirect indicator of total body iron storage. The efficacy of these two iron indices for anemia in chronic kidney disease cases remains indeterminate. [4]. In this context, hepcidin can serve as a superior biomarker for anemia in chronic kidney disease cases compared to conventional iron indices, as it fundamentally reduces serum iron availability by restricting iron efflux from whole-body iron storage. [5]. Current research indicates that hepcidin is a primary factor contributing to disturbances in the metabolism of iron in chronic kidney disease and anemia. [6, 7] It modulates systemic iron balance by reducing intestinal iron absorption and iron production from macrophages, hepatocytes, and enterocytes, resulting in hypoferremia and restricting iron availability for erythropoiesis. [6] Hepcidin levels rise in chronic kidney disease because of inflammation and decreased renal clearance. [7]. Serum hepcidin-25 concentrations, assessed through liquid chromatography–tandem mass spectrometry, demonstrated a negative correlation with hemoglobin levels in cases with non-dialysis-dependent chronic kidney disease (NDD CKD). Conversely, an additional investigation reported elevated hepcidin and other inflammatory markers in cases with early CKD, yet serum hepcidin wasn't associated with Hgb concentrations. The variation in findings can be attributed to the enzyme-linked immunosorbent assay utilized for measuring hepcidin or to a different case population. Resistance to erythropoietin in chronic kidney disease may result from elevated levels of hepcidin and iron deficiency. [8] Although hepcidin plays an essential part in iron metabolism, there is limited information associating hepcidin to anemia in stage four chronic kidney disease, and the challenges surrounding this relationship remain contentious. [9]

The goal of this work was to assess the role of hepcidin as a predictor for metabolism of iron disorders in stage 4 CKD cases with anemia and to evaluate the role of hepcidin as a predictor for inflammation in stage four chronic kidney disease cases with anemia.

Patients and methods

This case-control cross-sectional observational research has been conducted on 64 cases with CKD stage 4 with anemia divided into four groups: Group (A): involved 16 cases with hemoglobin levels below 6 g/dl; Group (B): included 16 cases with hemoglobin concentrations of 6-9 gram per deciliters; Group (C): included 16 cases with hemoglobin concentrations above 9-11 gram per deciliters; and Group (D): included 16 healthy subjects as a control group at **the** Nephrology Unit of the Internal Medicine Department in Assiut University Hospitals from April 2020 till July 2022.

Inclusion criteria: Adult cases (more than eighteen years) with anemia and stage four chronic kidney disease showing a glomerular filtration rate (GFR) of 15-29 milliliters per minute per 1.73 square meters.

Exclusion criteria: Subjects received blood transfusion within the previous six months, surgical history within the previous three months, malignancy, and advanced liver cirrhosis.

Ethical consideration: The investigation has been performed in accordance with the Declaration of Helsinki and attained acceptance from the Ethics Committee of the Faculty of Medicine, with the IRB number. The research's aim was clarified to all subjects, and written informed consent has been acquired. The research protocol has been registered on clinicaltrials.gov under NCT04102319.

Each patient was subjected to a full history and clinical examination, an assessment of glomerular filtration rate (GFR), laboratory investigations, and serum hepcidin was evaluated by standard ELISA kits.

Sample collection

Serum: Permit samples to clot for two hours at ambient temperature or overnight at two to eight degrees Celsius prior to centrifugation for twenty minutes. at 1000×g at two to eight degrees Celsius, obtain supernatant to conduct the assay.
Plasma: Obtain plasma utilizing heparin or EDTA as an anticoagulant. Centrifuge samples for fifteen minutes at 1000×g at two to eight degrees Celsius in thirty minutes of collection. Isolate supernatant to conduct the assay.
Reagent preparation: Allow all reagents to equilibrate to room temperature (between eighteen and twenty-five degrees Celsius) prior to utilization. When the kit can't be fully utilized in one assay, remove only the necessary reagents and strips for the present trial and store the remaining strips in the specified conditions.
Wash Buffer: Dilute thirty milliliters of concentrated wash buffer with 720 milliliters of distilled or deionized water to yield 750 milliliters of wash buffer. Attention: When crystals have developed in concentrate, heat it in a forty-degree Celsius water bath and stir gently till the crystals are entirely dissolved.
Standard working solution: Subject the standard to centrifugation at 10,000×g for one minute. Introduce 1.0 milliliters of reference standard and sample diluent, allow it to rest for ten minutes, and invert gently multiple times. Once it has completely dissolved, mix it carefully with a pipette. This reconstitution yields a functional solution of four thousand picograms per milliliter (or add one milliliters of reference standard and sample diluent, allow it to stand for one to two minutes, and subsequently mix carefully with a low-speed vortex mixer). Bubbles produced throughout the vortex can be eliminated by centrifugation at a comparatively low speed. Subsequently, conduct serial dilutions as required. The suggested dilution gradient is as follows: 4000, 2000, 1000, 500, 250, 125, 62.5, and 0 picograms per milliliter.
Dilution method: Utilize seven EP tubes and introduce five hundred microliters of reference standard and sample diluent into every one of them. Transfer five hundred microliters of the four thousand picogram per milliliter working solution to the 1st tube and mix to create a two thousand picogram per milliliter working solution. Transfer five hundred microliters of solution from the initial tube to the subsequent one as per this instruction. The illustration provided below serves as a reference. The final tube is deemed a blank. Don’t transfer the solution from the previous tube utilizing a pipette.
Biotinylated Detection antibody working solution: Determine the necessary amount prior to the experiment (one hundred microliters per well). In preparation, a quantity slightly exceeding the estimated amount ought to be prepared. Centrifuge the concentrated biotinylated detection antibody at 800×gram for one minute; subsequently, dilute the 100× concentrated biotinylated detection antibody to a 1× working solution using biotinylated detection antibody diluent (concentrated biotinylated detection antibody: biotinylated detection antibody diluent = 1:99).
Horseradish peroxidase conjugate working solution: Determine the necessary amount prior to the experiment (one hundred microliters per well). In preparation, a quantity slightly exceeding the determined amount ought to be ready. Centrifuge the concentrated horseradish peroxidase conjugate at 800 ×gram for one minute; subsequently dilute the one hundred × concentrated horseradish peroxidase conjugate to a 1× working solution using horseradish peroxidase conjugate diluent (concentrated horseradish peroxidase conjugate: horseradish peroxidase conjugate diluent = 1:99).
Assay technique (A brief assay technique is on the 12th page.)
Identify wells for the diluted standard, sample, and blank. Dispense one hundred microliters of every dilution of the standard, sample, & blank into the designated wells (it’s desirable to assay all standards and samples in duplicate). Apply the sealer included in the kit to plate. Incubate

for ninety minutes at thirty-seven degrees Celsius. Attention: Solutions ought to be introduced at the bottom of the micro-ELISA plate well, minimizing contact with the interior wall for preventing foaming. Decant the liquid from each well without washing. Promptly incorporate one hundred microliters of biotinylated. Detection antibody working solution for every well. Seal with the plate sealer. Incubate for one hour at thirty-seven degrees Celsius. Remove the solution from every well and add 350 microliters of wash buffer to every well. Soak for one to two min, then aspirate or decant the solution from every well and gently pat it dry with clean absorbent paper. Repeat this washing procedure three times. It is important to remember that a microplate washer may be utilized in this & subsequent washing steps. Utilize tested strips directly following the washing procedure. Prevent wells from becoming dry. Dispense one hundred microliters of horseradish peroxidase. Conjugate working solution into every well. Seal with plate sealer. Incubate for twenty minutes at thirty-seven degrees Celsius. Decant solution to every well and repeat the washing technique 5 times as performed in step three. Introduce ninety microliters of substrate reagent into every well. Seal with a new plate cover. Incubate for around fifteen minutes at thirty-seven degrees Celsius. Shield plate from illumination. Attention: The reaction time may be adjusted in accordance with the observed color alter, with however it is limited to thirty minutes. Preheat the microplate reader for approximately fifteen minute prior to optical density measurement. Introduce fifty microliters of stop solution into every well. It is essential that the stop solution be added in the identical sequence as the substrate solution. Simultaneously establish the optical density (OD value) of every well utilizing a micro-plate reader calibrated to 450 nanometers.

Estimation of results

Estimate the average of the duplicate evaluations for every sample and standard. Following that, subtract the average OD of the zero standard. Draw a 4-parameter logistic curve on log-log graph paper, with optical density values on the y-axis and standard concentration on the x-axis. When the samples were thoroughly diluted, concentration derived from the standard curve had to be multiplied by the dilution factor. When the optical density of the sample exceeds the upper limit of the standard curve, it is necessary to retest it with a suitable dilution. The actual concentration equals the calculated concentration multiplied by the dilution factor.

Results

An insignificant difference was discovered among the four examined groups according to sex and age (p-value was >0.05) **(Table 1).**

A significant difference was discovered among the four examined groups regarding s. creatinine and GFR (p-value was <0.001) **(Table 2).**

The mean hemoglobin increased steadily from group 1 to group 3. The control group showed a significant rise in Hb. Comparing with group one, group two, group three, and group four. A significant difference was discovered regarding HCT among the four groups with each other (except between groups 2 & 3) **(Table 3).**

A significant difference was discovered among the four examined groups according to s. iron (p-value was <0.001), and a pairwise comparison showed that the serum iron was significantly decreased in group one, group two, group three when comparing with group four (control group). A significant difference was discovered among the four examined groups according to TSAT (p-value was <0.001), and pairwise comparison showed that the TSAT was significantly reduced within group one, group two, group three if comparing with group four (control group). Pairwise comparison showed that the transferrin was significantly reduced within group 2, group 1, and group 3 if comparing with group 4 (control group). Pairwise comparison showed that the TIBC was significantly reduced within group 2, group 1, and group 3 if comparing

with group 4 (control group). Pairwise comparison showed that the ferritin was significantly reduced within group 2, group 1, and group 3 if comparing with group 4 (control group) **(Table 4).**

A comparison of levels of CRP among groups (one versus four, two versus four and three versus four) showed that the elevation in CRP levels was statistically significant **(Table 5).**

Pairwise comparison showed that the hepcidin was significantly high within group one, group two, and group three when compared to group four (control group). Also. Hepcidin level was significantly greater in group one than group two and group three **(Table 6).**

Using ROC curve, it has been demonstrated that: hepcidin may be utilized to discriminate among anemic groups and control group at a cutoff level of > 6.6 ng/ml, with 100% specificity, 93.7% sensitivity, 94.07% NPV and 100% PPV (AUC = 0.996 & p-value < 0.001) **(Table 7).**

Table (1): General characteristics among the examined groups.

		Group (1) (number= sixteen)		Group (2) (number= sixteen)		Group (3) (number= sixteen)		Group (4) (number= sixteen)		Test value	P-value
		No.	%	No.	%	No.	%	No.	%		
Gender	**Male**	12	75.0%	7	43.8%	8	50.0%	6	37.5%	X^2= 5.193	0.158
	Female	4	25.0%	9	56.3%	8	50.0%	10	62.5%		
Age (years)	**Mean± SD**	51.63± 9.39		53.44± 10.39		47.0± 8.69		43.31± 14.72		F= 2.744	0.051
	Median	52.0		55.5		45.0		41.5			
	Range	38.0- 67.0		37.0- 71.0		33.0- 63.0		35.0- 45.0			

p-value not more than 0.05 is deemed as statistically significant, p-value not more than 0.01 is deemed as highly statistically significant, SD: standard deviation, analysis performed by X2: Chi-Square Test & F: One-Way ANOVA Test.

Table (2): Renal function tests in the studied groups.

		Group (1) (number= sixteen)	Group (2) (number= sixteen)	Group (3) (number= sixteen)	Group (4) (number= sixteen)	Kruskal-Wallis Test	
						Test	P-value
S. creatinine (mg/dl)	**Mean± SD**	3.91± 1.21	4.29± 4.70	4.36± 1.72	0.91± 0.14	35.80	**<0.001**
	Median	3.45	4.70	3.75	0.91		
	Range	2.60- 6.50	2.0- 6.10	2.20- 7.0	0.70- 1.20		
GFR (ml / min per 1.73 m2)	**Mean± SD**	22.38± 3.50	19.95± 3.76	20.90± 5.24	112.33± 19.54	37.07	**<0.001**
	Median	22.90	19.05	20.35	105.0		
	Range	15.30- 27.70	16.0- 27.10	15.70- 26.60	90.60- 163.10		

analysis performed by X^2: Chi-Square Test& KW: Kruskal-Wallis Test.

Table (3): Hemoglobin and HCT in the studied groups.

		Group (1) (number= sixteen)	Group (2) (number= sixteen)	Group (3) (number= sixteen)	Group (4) (number= sixteen)	Kruskal-Wallis Test	
						Test	P-value
Hemoglobin (g/dl)	**Mean± SD**	5.79± 0.10	8.59± 0.23	9.36± 1.51	12.86± 1.02	53.62	**<0.001**
	Median	5.80	8.60	9.60	12.80		
	Range	5.60- 5.90	8.10- 8.90	5.60- 10.90	11.70- 14.40		

HCT	**Mean± SD**	17.86± 1.16	26.01± 1.04	25.54± 10.21	38.38± 2.59	48.03	**<0.001**
	Median	17.88	26.15	29.0	38.45		
	Range	17.0- 22.0	23.0- 27.5	0.31- 34.0	35.0- 42.0		

Table (4): Iron profile in the studied groups.

		Group (1) (number= sixteen)	Group (2) (number= sixteen)	Group (3) (number= sixteen)	Group (4) (number= sixteen)	Kruskal-Wallis Test	
						Test	P-value
S. iron (Ug/dL)	**Mean**	44.74	28.55	21.83	77.99	30.31	**<0.001**
	± SD	46.09	16.56	10.62	23.47		
	Median	26.50	27.50	21.95	78.80		
	Min.	7.50	13.40	8.80	42.50		
	Max.	184.00	81.00	45.20	119.00		
Tsat (%)	**Mean**	22.63	15.12	19.70	26.55	10.05	**0.018**
	± SD	22.15	8.04	20.66	11.67		
	Median	16.93	12.35	14.64	23.65		
	Min.	2.60	4.20	3.40	11.13		
	Max.	90.20	31.89	74.00	51.29		
Transferrin (g/l)	**Mean**	1.38	2.01	1.24	2.01	22.95	**<0.001**
	± SD	.51	2.51	.43	.37		
	Median	1.50	1.49	1.33	1.98		
	Min.	0.40	0.50	0.29	1.42		
	Max.	1.80	11.10	1.72	2.60		
TIBC (ug/dl)	**Mean**	187.81	208.00	279.31	309.56	25.83	**<0.001**
	± SD	63.97	78.90	303.45	45.72		
	Median	204.50	209.50	221.50	303.25		
	Min.	87.00	110.00	90.00	232.00		
	Max.	289.00	428.00	1400.00	382.00		
Ferritin (ng/ml)	**Mean**	581.25	436.44	601.93	134.32	14.77	**0.002**
	± SD	455.96	373.31	442.04	62.71		
	Median	458.95	364.97	405.00	123.61		
	Min.	1.70	31.97	93.00	63.24		
	Max.	1265.00	1265.00	1265.00	314.49		

Table (5): CRP in the examined groups.

		Group (1) (number= sixteen)	Group (2) (number= sixteen)	Group (3) (number= sixteen)	Group (4) (number= sixteen)	Test value	P-value
CRP (mg/l)	**Mean± SD**	59.13± 71.45	34.61± 41.03	40.19± 49.33	15.16± 26.34	KW= 9.34	**0.025**
	Median	28.0	12.35	26.5	3.0		
	Range	2.0- 196.0	3.0- 128.0	2.0- 188.0	2.0- 6.6		

Table (6): Hepcidin in the studied groups.

		Group (1) (number= sixteen)	Group (2) (number= sixteen)	Group (3) (number= sixteen)	Group (4) (number= sixteen)	Test value	P-value
Hepcidin (ng/ml)	**Mean± SD**	36.19± 9.49	21.53± 8.25	21.98± 10.41	4.96± 1.94	KW= 44.03	**<0.001**
	Median	39.0	20.25	21.5	4.80		
	Range	16.90- 49.0	10.20- 38.70	7.70- 48.0	3.9-6.6		

Table (7): Diagnostic performance of hepcidin.

	Cut off	Area under curve	Sensitivity	Specificity	positive predictive value	negative predictive value	p-value
Hepcidin	**>6.6**	**0.996**	**93.7%**	**100%**	**100%**	**94.07%**	**< 0.001**

Discussion

Insignificant variance was discovered among the four studied groups regarding sex and age (p-value was >0.05). A significant variance was discovered among the four examined groups according to s. creatinine and GFR (p-value was <0.001). The mean hemoglobin increased steadily from group 1 to group 3. Control group demonstrated significant rise in Hb. Comparing with group one, group two, group three, and group four. A significant variance regarding HCT among the four groups with each other (except between groups 2 & 3).

Our study agrees with **El-Toukhy et al. [10]**, who reported levels of hemoglobin (11.41 ± 0.53 grams per deciliter versus 14.23 ± 0.75 grams per deciliter, p-value = 0.000) demonstrating statistically significant reductions in hemodialysis kids as comparing with healthy control group.

A significant difference has been discovered among the four examined groups according to serum iron (p-value was <0.001), and pairwise comparison showed that the serum iron was significantly decreased within group one, group two, and group three compared to group four (control group). A significant difference has been discovered between the four studied groups regarding TSAT (p-value was <0.001), and a pairwise comparison showed that the TSAT was significantly decreased in group one, group two, and group three when compared to group four (control group). Pairwise comparison showed that the transferrin was significantly reduced in group one, group two, and group three if compared to group four (control group). Pairwise comparison showed that the TIBC was significantly reduced in group one, group two, and group three if compared to group four (control group). Pairwise comparison showed that the ferritin was significantly reduced in group one, group two, and group three if compared to group four (control group).

In concordance with our findings, **El-Toukhy et al. [10]** reported that serum iron concentrations (65.62 ± 0.62 milligram per liter versus 90.4 ± 0.73 milligram per liter, p-value = 0.000) and serum TIBC (234 ± 0.67 milligram per liter versus 258 ± 0.89 milligram per liter, p-value = 0.000) demonstrated a statistically significant reduction in kids with hemodialysis in comparison with healthy controls. Statistically significant elevations in serum ferritin concentrations (968.10 ± 5.3 nanograms per milliliter versus 674.00 ± 4.56 nanograms per milliliter, p-value = 0.000) have been observed in HD kids relative to healthy controls.

A comparison of concentrations of CRP among groups (one versus four, two versus four and three versus four) showed that the elevation in CRP levels was statistically significant.

The C-Reactive protein is a positive acute phase reactant correlated with both chronic and acute inflammation resulting from many different illnesses, including infections, non-infectious inflammatory conditions, and neoplasms. Standard references, to be unequivocally approved, vary based on the laboratory, gender, race, and age. Significant clinical inflammation should be considered if the concentration of CRP exceeds ten milligrams per liter (1 milligram per deciliter). A CRP level of 3–10 milligrams per liter (0.3–1 milligram per deciliter) indicates low-grade inflammation [11].

Pairwise comparison showed that the hepcidin was significantly increased in group one, group two, and group three if compared to group four (control group). Also. Hepcidin level was significantly greater in group one comparing with group two and group three.

In accordance with our findings **Tsuchiya et al.** [12] reported that Hepcidin is a crucial regulator of iron balance, and elevated serum hepcidin concentrations induce iron blockade and anemia in chronic conditions. Cases with CKD and anemia demonstrate higher serum hepcidin levels, which maybe result in anemia in CKD and to erythropoiesis-stimulating agent hypo-responsiveness. Serum hepcidin assessments in dialysis cases can't offer superior diagnostic utility compared to serum ferritin measurements.

Furthermore, **Al-Barshomy et al.** [13] reported that plasma hepcidin concentrations have been elevated in maintenance hemodialysis cases compared to healthy control participants. Additionally, a significant and independent positive association was observed among hepcidin and both serum ferritin concentrations and KT/V. A significant negative association was discovered among serum hepcidin concentrations and EPO dosage.

The ROC curve demonstrated that hepcidin effectively differentiates among anemic groups and the control group at a cutoff level exceeding 6.6 nanograms per milliliter, exhibiting 93.7% sensitivity, 100% specificity, 100% PPV, and 94.07% NPV (AUC = 0.996 & p-value below 0.001).

In line with our findings, **Han et al.** [14] stated that the identification of serum hepcidin in diagnosing anemia of chronic disease (ACD) yields a combined sensitivity of 0.94 and a combined specificity of 0.85, demonstrating the efficacy of serum hepcidin in ACD diagnosis and suggesting a low misdiagnosis rate.

Also, **Bârsan et al.** [15] revealed that in iron deficiency anemia, there is high ferroportin and low hepcidin expression in erythroblasts and macrophages, whereas the reverse is observed in of ACD. In regression analysis, elevated ferritin and Hep25 expected hepcidin expression (R^2 = 0.48; P-value < 0.0001), whereas decreased ferritin and Hep25 predicted ferroportin expression (R^2 = 0.29; P-value = 0.003) by erythroblasts; inflammation had no effect. In ROC analysis, serum ferritin and hepcidin demonstrated comparable moderate efficacy in distinguishing iron deficiency anemia from anemia of chronic conditions (area under curve 0.63, 95% confidence interval 0.47–0.79 and 0.76, 95% confidence interval 0.61–0.90, correspondingly).

Conclusion

Hepcidin was a good predictor for iron metabolism disorders and inflammation in stage 4 CKD patients with anemia. Hepcidin and CRP levels were the highest among patients with Hgb levels below 6 gm/dl. Hepcidin level correlated positively with the increase of ferritin in all patients.

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