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Relationship Between Hepcidin, Ferritin and C-Reactive Protein in Hemodialysis Patients

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ABSTRACT

Objective: Uremia is a state of heightened inflammatory activation. This might have an impact on several parameters including those used in the management of anemia as ferritin, serum iron, transferrin saturation, C-reactive protein (CRP) and hepcidin levels. In spite of this complexity the existing data indicate that hepcidin has an advantage over ferritin in guiding treatment of anemia in patients with chronic kidney disease (CKD) as it directly reflects iron availability and the status of iron homeostasis.

Aim of the study: was to determine serum hepcidin levels in maintenance haemodialysis (HD) patients and to investigate its relation to ferritin and markers of inflammation as C-reactive protein.

Subjects and methods: This study was conducted on 40 maintenance haemodialysis patients and 20 age-matched apparently healthy controls from October 2015 till February 2016 at the Haemodialysis Departement, National Institute of Urology and Nephrology (NIUN). Creatinine, albumin, hemoglobin, leucocytic count, CRP, hepcidin and ferritin were measured.

Results: Serum ferritin and hepcidin levels were significantly higher in HD patients compared with controls (825.67 ± 956.52 ng/ml and 9.2 ± 4.2 ng/ml vs 85.1± 63.35 ng/ml and 0.75 ± 0.39 ng/ml respectively) ($p< 0.001$).There was significant difference in CRP in HD patients compared with controls (4.28 ± 3.7mg/L vs 1.35±1.04mg/L respectively)($p<0.05$).There were insignificant positive weak correlations between serum levels of hepcidin and ferritin ($r = 0.05$, $P = 0.74$).

Conclusion: Serum hepcidin levels are increased in HD patients and, hence, could be used in the evaluation of anemia in such patients. Serum hepcidin provides useful information about the level and availability of iron during inflammation as compared with traditional markers of iron status. Availability of the ELISA assay for serum hepcidin will facilitate the routine measurement of hepcidin in clinical practice.

Keywords: Hepcidin, Ferritin, Hemodialysis, C-Reactive Protein.

INTRODUCTION

Anemia is commonly seen in all stages of renal disease but is much more pronounced in patients with end-stage renal disease (ESRD)[1].Patients with anemia due to chronic kidney disease (CKD) are at increased risk of hospitalization, increased length of hospital stay, reduced quality of life and higher mortality[2].The main causes of anemia in those patients are decreased erythropoietin (EPO) production, chronic inflammation, shortened half life of erythrocytes and iron deficiency[3].

Anemia can be corrected effectively using erythropoiesis-stimulating agents (ESA). However, a considerable proportion of patients exhibit a suboptimal response to ESA, and iron deficiency has been identified as the major cause of this hypo responsiveness [4].

Because of accelerated erythropoiesis driven by the ESA treatment (coupled with the ongoing uremia and dialysis-related iron losses), ESRD patients on ESA are at high risk of developing iron-restricted erythropoiesis because the rate at which iron is released from stores and delivered to the bone marrow fails to match the increased iron demand. This limited availability of iron to bone marrow can be corrected effectively by intravenous iron therapy, which improves hemoglobin (Hb) response[5].On the other hand, the inflammation frequently seen in dialysis patients may also contribute to iron-restricted erythropoiesis by reducing the release of stored iron from the reticuloendothelial system to circulating trans-ferrin, a condition that, unlike iron depletion, reduces the likelihood and extent of response to intravenous iron administration[6].

The observation that polymorph nuclear leucocytes from patients on maintenance hemodialysis (MHD) had two- to three-times the iron content as leucocytes of healthy subjects may reflect the defective regulation of iron transport proteins. The accurate identification of patients who would benefit from iron therapy has relevant clinical and economic implications, as it enables a better response to ESA, while avoiding the risks associated with overzealous iron therapy[7].Unfortunately, the laboratory tests used to evaluate iron status have revealed a suboptimal accuracy in identifying cases that will respond to intravenous iron,[8] as their relationships with iron status tend to be confounded by other factors, such

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as inflammation as in the case of ferritin, transferrin saturation (TSAT) and the percentage of hypochromic red blood cells (%Hypo),[9] or erythropoietin activity as in the case of soluble transferrin receptors (sTfR)[10]. (Nemeth et al.(2003) stated that a small peptide known as hepcidin, produced by hepatocytes circulates in the plasma and plays a central role in regulating the iron status in the body[11]. It binds to ferroportin, a cellular iron export channel protein, causing it to be internalized and degraded in lysosomes and preventing the efflux of iron from iron-exporting tissues into the plasma[12].Excess of hepcidin leads to dysregulation of iron metabolism in chronic kidney disease (CKD) patients[13]. Production of hepcidin is induced by excess iron stores and by inflammation, and is suppressed by erythropoietin activity[14]. It has been hypothesized that measuring serum levels of hepcidin may be useful as an additional tool for predicting and monitoring the need for iron supplementation. Elevated serum levels of the bioactive 25-amino acid hepcidin isoform, hepcidin-25 (Hep-25), have been consistently reported in dialysis patients,[15] probably due to the combination of an impaired renal excretion and an increased formation secondary to inflammation and iron overload[16]. Because Hep-25 blocks iron release from the macrophages, its increase may contribute to the disordered iron homeostasis and ESA resistance in uremia by limiting iron availability for erythropoiesis[17].

The present study was conducted to determine serum hepcidin levels in maintenance hemodialysis patients using the ELISA method and to investigate its relation to ferritin and markers of inflammation as C-reactive protein.

SUBJECTS AND METHODS

Subjects

This study was conducted on 40 maintenance hemodialysis patients and 20 age-matched apparently healthy controls from October 2015 till February 2016 at the Hemodialysis Department, National Institute of Urology and Nephrology (NIUN). Inclusion criteria for the maintenance hemodialysis patients were males and females aged > 18 years and inception of maintenance hemodialysis ≥ 6 months (three times per week for 4h per session) preceding the study and a baseline hemoglobin (Hb) level > 10 g/dl. Exclusion criteria were previous treatment with immunosuppressive drugs, clinical signs of acute infection, active inflammatory disease, liver disease, any malignancy, evidence of blood loss (gastrointestinal bleeding, trauma, etc.)

METHODS

Venous blood samples were collected midweek from maintenance hemodialysis patients, after an overnight fasting, immediately before the session of hemodialysis. For healthy control subjects, blood was also drawn from a peripheral vein after an overnight fasting. The samples were drawn into plain vacutainer tubes and centrifuged at 3500 rpm for 15 minutes, aliquoted and stored at –20°C until analysis. Evaluation of hepcidin was done using the commercially available human hepcidin Enzyme linked-Immunosorbent Assay (ELISA) kit (Uscnlife,Wuhan Elaab Science Co..LTD) [18]. Evaluation of ferritin was done using kits for VIDAS (bioMerieux SA-France) and measured by Enzyme Linked Fluorescent Assay (ELFA) technique (Minividas, bioMerieux, France) [19]. Serum levels of albumin and creatinine were measured by automated Dimension RXL, Dade Behering, USA. CRP was done using Turbox apparatus Orion Diagnostica Espoo Finland. Hemoglobin and white blood cell count (WBC) were determined by automated procedure using Cell Dyne 1800 apparatus Abbott Diagnostic, USA.

The study was done after approval of ethical board of National Institute of Urology and Nephrology and an informed written consent was taken from each participant in the study.

Statistical analysis

Analysis of data was performed using SPSS 21 for Windows. Description of variables were presented as follows: Description of numerical variables in the form of mean, Standard Deviation (SD), Median, 25^{th} and 75^{th} percentiles. Description of categorical variables in the form of numbers (No.) and percent's (%). Parametric tests for numerical data with normal distribution except WBCs (not normally distributed). Student T-test for two independent samples for comparison between numerical variables. Mann–Whitney U test for comparing between two groups of independent variables (WBCs). Chi-Square test (X2) for comparison between categorical variables. Results were expressed in the form of P-values. P-value ≤ 0.05 for assessment of significance.

For binary correlation, Pearson correlation test in most of cases and Spearman correlation tests were used in case of nonparametric variables. Results were expressed in the form of correlation coefficient (r) and P-values.

RESULTS

The study was conducted on 40 maintenance HD patients (23 males,17 females with mean age of 44

.22 ±13.76 years) and 20 age-matched healthy controls (8 males,12 females with mean age of 31.1±10. 18 years) (table 1).

Hemoglobin, serum albumin and creatinine levels were significantly higher in hemodialysis patients compared with healthy controls (11.23 ± 0.87g/dl,3.35 ±0.25 g/dl and 10.33 ± 2.35 mg/dl vs 13 ± 0.46 g/dl,3.7± 0.21 g/dl and 0.62 ± 0.13 mg/dl respectively) (p< 0.001) (table 1).

Total WBCs count was insignificantly higher in HD patients compared with healthy controls(7.28 ± 2.17 10^3/cmm vs 6.73 ± 1.48 10^3/cmm (p 0.59)(table 2).

Serum ferritin and hepcidin levels were significantly higher in HD patients compared with healthy controls (825.67± 956.52 ng/ml and 9.2 ± 4.2 ng/ml vs 85.1±63.35 ng/ml and 0.75 ± 0.39 ng/ml respectively) (p< 0.001)(table 2,figure 1 and 2).

There is significant increase in CRP in HD patients compared with healthy controls (4.28 ± 3.7mg/L vs 1.35±1.04mg/L respectively)(p<0.05)(table 2).

There were insignificant positive weak correlations between serum levels of hepcidin and Hb (r = 0.14, p = 0.38) and WBCs (r = 0.14, P = 0.1)(table 3, figure 3,4). There were insignificant negative weak correlations between serum levels of hepcidin and albumin (r =- 0.16, P = 0.31) (table 3,figure 5).

There were significant negative moderate correlation between serum levels of hepcidin and creatinine (r =- 0.31, P = 0.05)(table 3,figure 6).

There were significant positive correlation between serum levels of hepcidin and CRP (r = 0.41, P < 0.05)(table 3).

There were insignificant positive weak correlations between serum levels of hepcidin and ferritin (r = 0.05, P = 0.74)(table 3,figure 7).

Table: 1 Demographic characteristic of patients and controls

Item / Subjects	controls	Patients	P value
Number	20	40	
Age	31.1±10.18	44.22±13.76	<.001(H.S.)
Hb (g/dl)	13.0±0.46	11.23±0.87	<.001(H.S.)
Albumin (g/dl)	3.7±0.21	3.35±0.25	<.001(H.S.)
Cr (mg/dl)	0.62±0.13	10.33±2.35	<.001(H.S.)

Table 2: Comparison between patients and controls as regards WBCs , Ferritin & Hepcidin

Item / Subject	Controls	Patients	P value
WBCs (×103/cmm)	6.73±1.479	7.28±2.17	0.59 (NS)
Ferritin (ng/ml)	85.1±63.35	825.67±956.52	<.001(H.S.)
Hepcidin (ng/ml)	0.75±0.39	9.2±4.12	<.001(H.S.)
CRP (mg/L)	1.35±1.04	4.28 ± 3.7	<0.05 (S)

Table 3: Univariate analysis of the association between serum hepcidin level and clinical and laboratory parameters in maintenance hemodialysis patients (n = 40)

Group: cases		Hepcidin ng/ml
Hb g/dl	Pearson Correlation "r"	0.141
	P value	0.386
Albumin g/dl	Pearson Correlation "r"	-0.164
	P value	0.313
Cr mg/dl	Pearson Correlation "r"	-0.312
	P value	0.05
WBCs10^3/cmm	Spearman's rho Correlation "r"	0.147
	P value	0.108
Ferritin ng/ml	Pearson Correlation "r"	0.05
	P value	0.74
CRP mg/L	Pearson Correlation"r"	0.41
	P value	<0.05

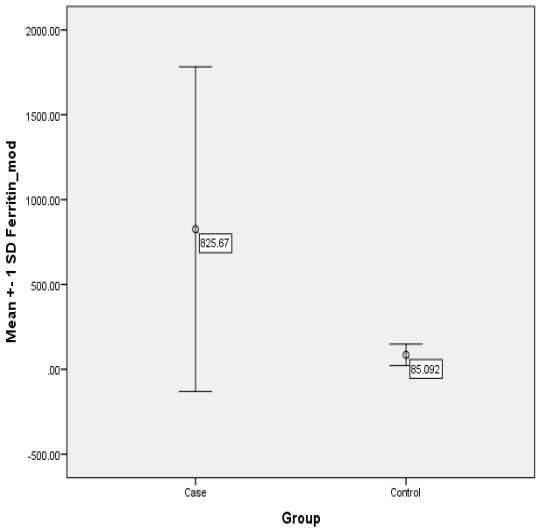

Figure 1: mean serum ferritin level in maintenance hemodialysis patients (n = 40) and healthy control subjects (n = 20)

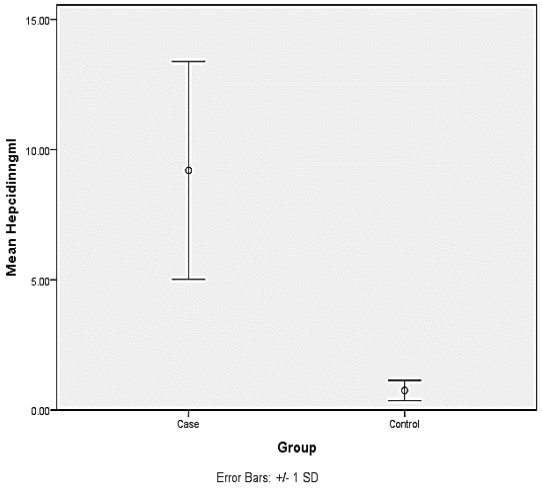

Figure 2: mean serum hepcidin level in maintenance hemodialysis patients (n = 40) and healthy control subjects (n = 20)

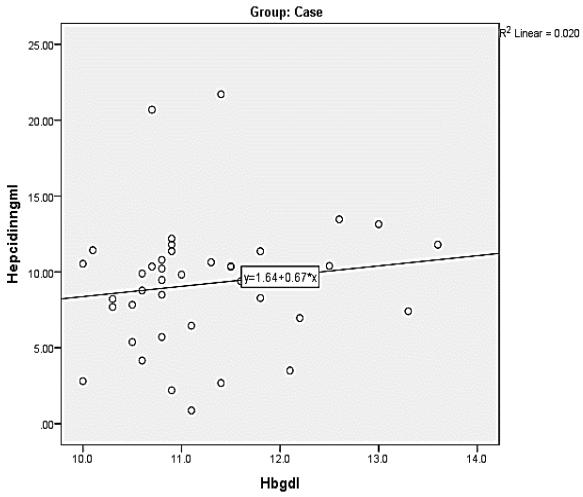

Figure 3: correlations between serum levels of hepcidin and Hb in maintenance hemodialysis patients (n = 40)

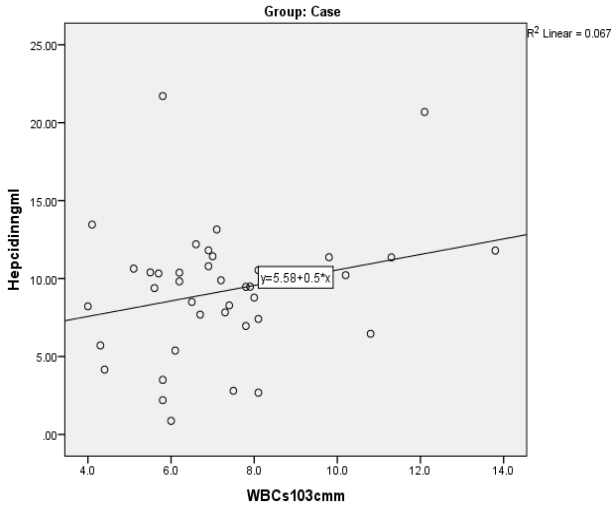

Figure 4: correlations between serum levels of hepcidin and WBCs in maintenance hemodialysis patients (n = 40)

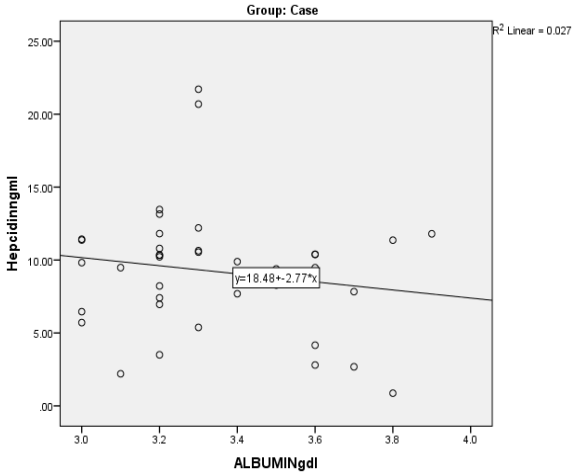

Figure 5: correlations between serum levels of hepcidin and albumin in maintenance hemodialysis patients (n =40)

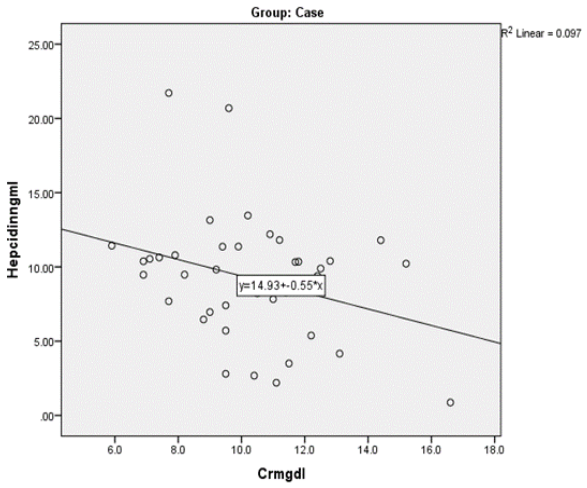

Figure 6: correlations between serum levels of hepcidin and Cr in maintenance hemodialysis patients (n = 40)

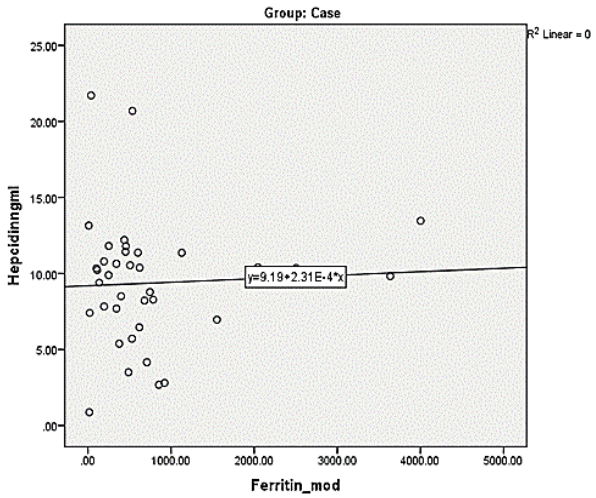

Figure 7: correlations between serum levels of hepcidin and ferritin in maintenance hemodialysis patients (n = 40)

DISCUSSION

Uremia is a state of heightened inflammatory activation. This might have an impact on several parameters including those used in the management of anemia. Ferritin, for example, is a marker of body iron stores, but it also increases in acute inflammation and therefore becomes less valuable as an indicator of iron status during inflammation[20].Serum iron and transferrin saturation are also influenced by inflammation. Inflammation also increases CRP and hepcidin levels,[21] but in spite of this complexity the existing data indicate that hepcidin has an advantage over ferritin in guiding treatment of anemia in patients with CKD as it directly reflects iron availability and the status of iron homeostasis, better than other conventional parameters[22].Hepcidin-20 and hepcidin-22 are its isoforms with unknown biological function (Coyne,2011)[23]

Hepcidin levels are regulated by iron status and erythropoietic activity[24].It is well documented that hepcidin levels are reduced by anemia and hypoxia and increased by inflammation[25].Renal anemia is considered as a special form of anemia of inflammation[26] .

The present study demonstrated that serum hepcidin levels were higher in HD patients than in healthy controls. This finding agrees with **Tessitore *et al.***[27] who found that the mean serum levels of the bioactive isoform Hep-25 were higher in 56 HD patients than in 57 controls and this is also consistent with **Xu *et al.***[28] and **Rubab *et al.*** studies.[29]

The concentration of serum hepcidin did not differ significantly in peritoneal dialysis when compared to HD patients[30].Genes regulating hepcidin expression have been discovered, and defects in them mostly resulted in iron overload.TMPRSS6 gene is the first gene regulating hepcidin and encodes a negative regulator of hepcidin expression. Any mutation in this gene would cause chronic iron-deficiency anemia[31].

On the other hand, we found that serum ferritin levels were elevated among our HD patients. Findings consistent to ours have been seen in a study on patients with CKD by **Yilmaz *et al.***[32]The situation in which the transferrin saturation (TSAT) is low and the serum ferritin is high is frequently seen among HD patients. High ferritin levels may be due to functional iron deficiency or reticule-endothelial blockade. This commonly seen paradox of high serum ferritin and low TSAT has made it desirable to look for a substitute of iron markers to predict better iron status of the patient[8].The diagnosis of iron deficiency using these markers is unproductive, as they can be affected by variables such as age, sex, inflammation and nutritional factors. **Sancho *et al.***[33] concluded that determining hepcidin concentrations together with conventional markers associated with iron metabolism improved the identification of patients with iron deficiency by 26.1%.

In this study in a cohort of stable prevalent HD patients, hepcidin levels were shown to have insignificant weak positive association with iron stores (as reflected by ferritin levels) and significant positive association with CRP as a marker of inflammation. **Ashby *et al.***[34] demonstrated that hepcidin levels (using a radioimmunoassay) were significantly elevated in HD patients, but did not correlate with ferritin which is in agreement with our results. Also, the levels of hepcidin showed insignificant correlation with serum ferritin level in 42 HD patients in **Rubab *et al.***[29].

In contrast to our study, **Fujita *et al.***[35] demonstrated that the serum ferritin level had a strong positive correlation with the hepatic levels of hepcidin mRNA expression. **Xu *et al.***[28] observed a significant and independent correlation between hepcidin and ferritin levels. This could be explained by the fact that the ferritin levels of the patients in our study are much higher than in their study. **Weerd *et al.***[36] and **Sany *et al.***[37] studies did not agree with our finding. **Weerd *et al.***[36],found that hepcidin levels were shown to be independently and positively associated with ferritin levels and ferritin was the strongest determinant of hepcidin

in 405 HD patients. The relation between hepcidin and ferritin was present irrespective of the level of inflammation. However, whether hepcidin is upregulated in response to increased ferritin levels cannot be concluded from their study. The difference between the former study and ours could be explained by the big cohort of patients in their study. **Sany *et al.*, study,**[37] confirmed a significant correlation between serum ferritin and hepcidin levels in 80 HD patients.

Hepcidin levels are likely to be higher in CKD patients due to limited hepcidin excretion, tissue iron overload and inflammation. Patients undergoing continuous dialysis are in a chronic inflammatory state. The effects of inflammation on the synthesis of hepcidin are well understood 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[38] . In this study, CRP was measured as the conventional marker of inflammation and was found to be significantly increased in HD patient when compared with controls. It is known that hepcidin synthesis is induced by inflammation, a process that is mediated by IL-6. As CKD is considered an inflammatory state, this positive correlation was expected[39].Our results are comparable to **Malyszko *et al.*,**[40]and **Samouilidou *et al.*,** [30] studies., which showed a correlation of hepcidin levels with CRP. However, **Zaritsky *et al.***[39]showed no correlation between hepcidin and CRP levels. This may be explained on the basis of differences in the half-lives of CRP and hepcidin. Another explanation may be the distinct features of the study population, i.e. stable maintenance HD patients with little or no dialysis-related inflammation, and stable Hb[27]. **Sasu *et al.***[41] has shown that comparison between hepcidin and CRP may serve as a quick and easy method for identifying the difference between iron deficiency, inflammation or mixed anaemia. In such a diagnostic scheme, negative CRP and low hepcidin would indicate iron deficiency, high CRP and high hepcidin would indicate inflammation, while high CRP and low hepcidin would indicate a mixture of inflammation and iron deficiency. **Przybyszewska *et al.***[42] found that hepcidin concentrations were similar in their elderly patients with anemia of chronic disease compared with those with iron deficiency anemia.

Zaritsky *et al.*[15] reported that being a very small molecule, hepcidin could be cleared efficiently by HD. The findings of the **Xu *et al.***[28] study showed that as no significant difference was observed in serum hepcidin levels in a single HD session. This was consistent with a study by **Ashby *et al.*,**[34] that showed absence of reduction following a standard dialysis session. The cause of this variability remains unclear, but might be attributable to differences in the membrane of the dialyzer, residual renal function or induction of hepcidin by the HD procedure[44].

Martinelli *et al.*[45] study reported that it was established a link between iron metabolism and insulin resistant states, including diabetes mellitus. **Li and his colleagues**[43] observed that serum hepcidin in HD patients with diabetic nephropathy was significantly higher than those with non diabetic nephropathy.

We found that hepcidin was significantly correlated with creatinine, but with negative insignificant correlation with albumin. This is in agreement with **Aydin *et al.***[46]. However the correlation between hepcidin and albumin is significantly negative.The difference in albumin is due to the small size of our study. We observed that in HD patients, the mean Hb was significantly lower than the controls. This is in agreement with **Samouilidou *et al.***[30] study in his 30 end-stage renal disease and 30 HD patients and **Rubab *et al.***[29] findings in his 42 HD patients.

We are aware that our study had some limitations, as it was a single-center study on a small sample of patients; therefore, it may be underpowered for evaluating the role of different biomarkers in predicting iron status. finally, as only a single determination of hepcidin was made, any variation that may have occurred over time cannot be taken into account. Further clarification of the correlation between hepcidin regulation and iron storage is needed.

CONCLUSION

Serum hepcidin levels are increased in HD patients and, hence, could be used in the evaluation of anemia in such patients. Serum hepcidin provides useful information about the level and availability of iron during inflammation as compared with traditional markers of iron status. Availability of the ELISA assay for serum hepcidin will facilitate the routine measurement of hepcidin in clinical practice.

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