THE ASSOCIATION BETWEEN LEVELS OF HEPCIDIN, IRON STATUS AND MICRO-INFLAMMATION MARKERS AMONG HAEMODIALYSIS PATIENTS

ABSTRACT

Hepcidin is a polypeptide that regulates iron homeostasis and could serve as an indicator of functional iron deficiency in patients with end-stage renal disease (ESRD); this may also aid in the assessment of patient's response to erythropoietin (EPO). The present study was aimed to investigate serum levels of hepcidin, iron status and inflammation markers such as C-reactive protein (CRP) in patients with ESRD on maintenance HD and to observe the correlation of serum hepcidin with conventional iron and inflammatory markers.

A total of 59 patients on maintenance HD were enrolled; 29 age and sex-matched healthy subjects were included as controls. Laboratory tests including complete blood count, creatinine, urea, albumin, BUN, serum hepcidin, serum ferritin, serum iron and CRP were performed. The serum hepcidin levels was measured by a competitive enzyme-linked immunosorbent assay (C-ELISA). Serum hepcidin levels were significantly higher in patients with ESRD than in the control group (63.7± 47.4 ng/mL :11.5± 26.3 ng/mL respectively $P<0.001$). The hemoglobin and serum iron levels in the patient group were significantly lower than in the control group. Higher feritine levels were found in hemodialysis patients (448.5± 710 ng/mL): (98.3 ± 83 ng/mL) of controls (P =0.01). A positive and significant correlation was observed between the values of serum hepcidin and CRP. Serum hepcidin and high-sensitivity C-reactive protein levels were significantly higher in maintenance haemodialysis patients (case =21.2± 28.6 mg/L :control =2.9 ± 2.7 mg/L, P=0.001) .

In conclusion, higher hepcidin levels are found in ESRD patients and serum hepcidin levels are associated with iron status and micro-inflammation (defined as hsCRP < 6mg/l, in maintenance haemodialysis patients). Also, our findings suggest that hepcidin might play a role in the pathophysiology of anemia associated with chronic diseases as ESRD. As well as, ELISA method for measuring serum hepcidin should facilitate the routine measurement of hepcidin in clinical practice.

Key words: Ferritin, Hepcidin, Hemodiylasis , ESRD, renal failure, association, Yemen

INTRODUCTION

Anemia is commonly seen in all stages of renal disease but is much more obvious in patients with end-stage renal disease (ESRD).[1] Patients with anemia due to chronic kidney disease (CKD) are at enlarged risk of hospitalization, increased length of hospital stay, lower quality of life and higher mortality.[2] The main causes of anemia in patients with CKD are chronic inflammation, decreased erythropoietin (EPO) production, iron deficiency, and shortened half life of erythrocytes.[3] Hepcidin is a peptide secreted by the liver that regulates plasma iron.[4] Hepcidin production decreases in the presence of hypoxia, iron deficiency, and ineffective erythropoiesis[5], whereas increased production is stimulated by increased plasma and stored iron.[6] Increased levels of hepcidin results in iron release in macrophages and decreased absorption of iron.[7] Hepcidin production is also increased by inflammation and high hepcidin concentrations limit iron availability for erythropoiesis, thus playing a major role in the anemia of inflammation and EPO resistance.[8] Hemodialysis (HD) is considered an inflammatory condition and increased serum hepcidin levels have been found in patients with ESRD on maintenance HD.[9] These raise levels in hemodialyzed patients could be due to functional iron deficiency anemia and low-grade inflammation.[10] Reticulo–endothelial blockade is seen during inflammation, which is mediated by hepcidin up-regulation, and results in inhibition of release of iron to transferring.[8,11] Hepcidin also be part of the cause of EPO resistance by regulating iron-restricted erythropoiesis and by its inhibitory effect on erythroid progenitor proliferation and continued existence.[8,11] 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 consequently becomes less valuable as an indicator of iron status during inflammation.[12] Serum iron and transferrin saturation are also inclined by inflammation. Inflammation also increases the C-reactive protein (CRP) and hepcidin levels[9] but in state 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.[13] The current study was designed to determine the values of these conventional markers of body iron stores, degree of inflammatory activation and serum hepcidin in patients with ESRD on maintenance HD and to compare them with normal controls.

SUBJECTS AND LABORATORY METHODS

Study Design and site: A case control comparative study was conducted to achieve the objectives of the study. The study included 88 individuals aged 20-45 years old and they divided in to two groups: **Group1:** Consist of 59 individuals who are end stage renal failure haemodialysis patients, we included only patients whom had maintenance haemodialysis for more than one year in nephrology and urology center in Al-Thawra University

Hospital- Sana'a City. Also pregnant women, patients with liver disease, and /or patients with Cardiovascular disease or any other chronic disease were excluded. **Group 2:** Consist of 29 healthy individuals attending the same Hospital for routine examination (volunteers), who don't suffer from any other chronic diseases. All Patients and controls agree to fill the written informed consent of the study.

Data collecting: Data regarding demographic, clinical characteristics, body mass index (BMI), and duration of dialysis were recorded in predesigned forms for all patients, also demographic , clinical characteristics, and body mass index (BMI) were recorded in predesigned forms for all controls.

Collection and storage of blood samples: Peripheral blood samples were collected in a vacationer tubes after an overnight fast, before a single session of haemodialysis for each patient. For controls also peripheral blood samples were collected in a vacationer tubes after an overnight fast for each controls. The whole blood samples were obtained using EDTA-tubes for measuring hematological markers for cases and controls. For serum Iron, serum ferritin, serum CRP and serum hepcidin 10 ml blood were collected in 10 ml capacity tubes containing Clot activator , then serum were separated by centrifuging, and stored in aliquots for cases and controls. Blood samples which occurred hemolysis would be discarded. Each serum sample was given a tracking number and stored at − 20 °C until further analysis.

Laboratory Tests: Hepcidin was tested in serum samples using manual enzyme-linked immunosorbent assay (ELISA) kit . Ferritin was tested in serum samples using automated enzyme-linked immunosorbent assay (ELISA) system. The tests were performed according to the manufacturer's instructions described in the assay procedure. Blood Iron, CRP, albumin, creatinine were tested in the samples using automated system (integra 400 close system). Also. Hb and white blood cell count (WBC) were determined by automated procedures.

Data analysis: The analysis of data was done by Epi Info version 6 statistical program (CDC, Atlanta, USA), where the chi-square ($\chi 2$) and probability value (p) was calculated for the test of significance by comparing the geometric mean ± SD of the serum level of hepcidin, ferritin, iron and etc among cases (HD patients) comparing with that of healthy controls. In addition, Odd's ratio (*OR*), 95% confidence interval (*95% CI)* were added to estimate the associated *OR* for high level of hepcidin, ferritin and iron etc. with HD patients comparing with that of healthy controls. A *p value* < 0.05 was considered significant.

Ethical approval

We obtained written consent from all cases and controls. Assent was taken from participants before collecting the specimens. The study proposal was evaluated and approved by the Ethics Committee of Faculty of Medicine and Health Sciences, Sana'a University.

RESULT

HD patients were counting 59 patients, 37 of them were males and 22 were females. The unmatched healthy controls of study were counting 29 healthy unmatched in age and sex with the patient group. The control included 17 males and 12 females. The Mean±SD of the patient's ages was 28.3±6.4 years and their ages ranged from 20-45 years, while the Mean±SD of the control's ages was 25±3.5 years and their ages ranged from 20-40 years. Also the BMI (Kg/m^2) Mean±SD of patients was 18.2±3.8 kg/m^2 lower than 24.1±2.83 kg/m^2 of the healthy controls (table 1). Table 2 shows the serum hepcidin and conventional markers of Iron status and markers of inflammation (CRP, WBCs) in hemodialysis patients and controls. The serum hepcidin levels were significantly (p=<0.001) higher in patients with ESRD on HD (Mean±SD= 63.77±47.4 ng/ml) compared to lower level in controls (Mean±SD=11.5±26 ng/ml). The Hb levels were significantly (p=<0.001) lower in patients (Mean±SD=7.97±1.4 g/dL) compared with healthy controls level (Mean±SD=15.55±1.3g/dL). The serum Ferittin (ng/ml) levels were significantly (p=<0.001) higher in patients (Mean±SD= 448.5±710 ng/ml) as compared with healthy controls level (Mean±SD was 98.3±83ng/ml). The serum CRP levels were significantly (p=<0.001) higher in patients (Mean±SD = 21.3±28.6 mg/L) as compared with healthy controls (Mean±SD =2.9±2.7mg/L). However no significant variations were observed in Serum Iron level and WBCs for both patients and controls. Table 3 shows the serum albumin and conventional markers of kidney function in hemodialysis patients and controls. All these markers were significantly (p=<0.001) abnormal in patients and normal in healthy controls (table 3). Table 4 shows the association of high level of hepcidin with conventional markers of iron status and marker of inflammation (CRP) among hemodialysis patients. In HD patients the rate of high level of hepcidin was 62.7%. There was no association between high level of hepcidin and high level of ferritin, but there was association with low level of Iron (*OR*=4.9) with non-significant result (*P*=0.11). Also there is association between high level of hepcidin and positive CRP (OR=1.2) and this result was not statistical significant (p=0.76). Table 5 shows the associated odds ratio of low level of serum iron, high level of serum hepcidin, high level of serum ferritin, and positive CRP, in hemodialysis patients comparing with healthy controls. The rate of high level of ferritin was 37.3% in HD patients comparing with 13.7% in controls. There was association between high level of ferritin in HD patients with positive CRP (*OR*= 3.7, *CI*=1.5-12, p=0.02). In addition there was association between low level of iron and HD patients (*OR*=1.5, *CI*=1.3-1.8, p=0.037). Also the risk difference to develop low level of Iron in HD comparing with controls is 36 times (*CI*=25-46). The rate of positive CRP was 52.5% in HD patients comparing with 3.4% in controls. In addition there was association between positive CRP and HD patients (*OR*=

31, *CI*=3.9 -243, *p*<0.001). Also the risk difference to develop positive CRP in HD comparing with controls is 46 times and this difference ranged from not less than 32 and up to 61 times. The rate of high level of hepcidin was 62.7% in HD patients comparing with 6.9% in controls. In addition there was association between high level of hepcidin and HD patients (*OR*= 22.7, *CI*=4.9 - 104, *p*<0.001). Also the risk difference to develop high level of hepcidin in HD comparing with controls is 49 times and this difference ranged from not less than 34 and up to 65 times. Table 6 shows the comparison of high (> 6mg/l) and low (<6 mg/l) CRP levels in relation to age, gender and hemodialysis duration for patients. The mean ± SD age of CRP positive patients was 33.4±5.8 years older than the mean ± SD age of CRP negative patients (26.5±7.1 years). Therefore there is association between age growing and occurring of positive CRP and this result was significant (p<0.001). Also, when we considered sex of the patients, there was significant association between male patients and positive CRP (p<0.05), that is mean male patients have more possibility to develop CPR positive than female patients. What is more, when we considered duration of hemodialysis and positivity of CRP, there was association between longer duration of hemodialysis and positive CRP. The mean ± SD duration of CRP positive patients was 4.9±0.6 years longer than the mean ± SD duration of CRP negative patients (3.8±0.4 years), but this variation was not statistically significant (p=0.08). Therefore there is association between long duration and occurring of positive CRP but not significant due to small sample size of the current study.

DISCUSSION

Hepcidin is now acknowledged to be the main iron regulatory hormone. It is a 25-amino acid peptide exclusively synthesized by the liver, initially identified as part of a search for novel antimicrobial peptides.[14,15] There was no indication that it had an additional role in iron metabolism until 2001, when mouse studies were published showing that hepatic hepcidin mRNA synthesis was induced by iron loading.[16,17] Also, Hepcidin levels are regulated by iron status and erythropoietic activity.[18] It is now well documented that hepcidin levels are reduced by anemia and hypoxia and increased by inflammation.[19] Renal anemia is considered a special form of anemia of inflammation .[20] The present study focused on the levels of serum hepcidin in patients with ESRD on maintenance HD for at least one year and their levels were then compared with controls.

In the present study, the enzyme-linked immunosorbent assay (ELISA) method was used for the detection of serum hepcidin levels and found that the levels were significantly (*p*<0.001) higher in patients with ESRD on HD in which the hepcidin Mean±SD level was 63.77±47.4ng/ml as compared with healthy controls (Mean±SD =11.5±26ng/ml) (table 2). Similar results were also reported by Zille *et al.* [21] in which the hepcidin Mean±SD level was 18.2 ± 2.8 ng/ml as compared with healthy controls in which the hepcidin Mean±SD level was 8.1 ± 2.3 ng/ml.[21] In present study results hepcidin levels were six –fold higher in patients of hemodialysis than the healthy controls (table 2). Also when the high level rate of hepcidin with hemodialysis considered there was significant odds ratio of high level of hepcidin with hemodialysis equal to 22.7 times comparing with that of controls (table 5). The current study result is higher than that reported by Jairam *et al.*[12] and Swinkels and Wetzels[13] in which hepcidin levels were only two- to three-fold higher in patients with hemodialysis comparing with controls. The high levels of hepcidin in hemodialysis patients can be explained by the fact that there are limited excretion of hepcidin by kidney or by dialysis in patients lead to elevation of hepcidin which cause tissue iron overload and inflammation.[22]

Among present study group of hemodialysis patients, a significant decreased levels of Hb was found in which Hb Mean±SD level was 7.97±1.4g/dl as compared with healthy controls (Mean±SD level=15.55±1.3g/dL, *p*<0.001) (table 2). However, in the current study there was non-significant decreased levels of the serum iron in among patients (Mean±SD=142.88±65ng/ml) as compared with healthy controls (Mean±SD=159.6±64ng/ml, *p*=0.35) (table 2). The present study findings are similar to that reported by Yilmaz *et al.*[23] in which they reported renal anemia in most hemodialysis patients but with significant decreased levels of the serum iron in the patients comparing with controls.

In the present study serum ferittin levels found to be significantly (*p*<0.001) higher in patients with ESRD on HD (448.5±710ng/ml) as compared with healthy controls (98.3±8.3ng/ml) (table 2) . These findings consistent to Yilmaz *et al.*[23] in which elevated ferittin was seen in patients with CKD. High ferittin levels may be observed in this disease because of functional iron deficiency or reticulo–endothelial blockade. This commonly seen paradox of high serum ferittin has made it desirable to look for a substitute iron marker to predict better iron status of the patient.[24] Various other studies also support that current markers of iron metabolism; like ferittin do not predict iron status effectively[24] and that this conventional marker have certain limitations.[6] The diagnosis of iron deficiency using this marker is unproductive, as it can be affected by variables such as age, sex, inflammation and nutritional factors. In another study, it was concluded that determining hepcidin concentrations together with conventional markers associated with iron metabolism improved the identification of patients with iron deficiency by 26.1%.[25]

In this study, CRP was measured as the conventional marker of inflammation and was found to be higher in patients than in controls, significantly (*p*<0.001) (21.3±28.6mg/dL: 2.9±2.7mg/dL) (table 2); and also positive CRP were found to be significantly correlated with high serum hepcidin (*OR*=31, *p*<0.001) (table 5). 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[26] The present study results are comparable to other studies on patients with renal failure, which showed a correlation of high hepcidin levels with positive CRP.[27] However, these results are different from the finding of Memoli[26] and Tovbin[28] in which no correlation was observed between hepcidin and CRP levels in CKD patients. This lack of correlation may be explained on the basis of differences in the half-lives of CRP and hepcidin.[28]

When high level (> 6mg/l) comparison with low level (<6 mg/l) of CRP in relation to hemodialysis in the current study, there was 52.5% of the patients had high (> 6mg/l) CRP level (table 6). This result is higher than that reported by Korevaar *et al.* [29] in which this prospective multicenter study on the impact of an HD session on CRP level showed an increase in CRP level in 25% of the patients during a dialysis session. Moreover, independent of the pre-dialysis CRP level, the change in CRP level during an HD session was associated with an increased mortality risk; an increase of 1 mg/L in CRP level was associated with a 9% raised mortality risk. The present study finding suggests that a 52.5% of our patient with an increase of CRP level will have a raised mortality risk than the low CRP level patients. CRP is a marker of inflammation, which involves a number of complex processes that can be induced by any trauma or infection. As soon as the inflammatory stimulus has been eliminated, the CRP level declines. The increase in CRP level observed in the present study could have been the result of such acute trauma or infection or a response to the dialysis. There was significant association between high CRP and older HD patients (<0.001) (table 6) in the present study, this is similar to that reported by Rashid *et al.* [27] in which the rate of positive CRP was higher in older ages of HD patients. Additionally, it has been reported that the occurrence of inflammation is higher in patients who were on dialysis for a longer time [30] but there was no significant association between CRP and length of dialysis in the current study (table 6).

CONCLUSION

Higher hepcidin levels are found in ESRD patients and serum hepcidin levels are associated with iron status and micro-inflammation (defined as hsCRP < 6mg/l, in maintenance haemodialysis patients). Also, our findings suggest that hepcidin might play a role in the pathophysiology of anemia associated with chronic diseases as ESRD. As well as, ELISA method for measuring serum hepcidin should facilitate the routine measurement of hepcidin in clinical practice.

RECOMMENDATION

A extensive information of hepcidin regulation will provide us with original tools for differential diagnosis, therapeutic regimes and monitoring of disorders of iron metabolism particularly in HD patients. However, a lot remains to be revealed on the biology and function of hepcidin. Its signaling pathways are as up till now to be defined. Additional studies are needed to define accurately the hepcidin role in iron metabolism homeostasis and its efficacy in the diagnosis and treatment of iron disorders. Also that more studies need to be performed regarding the role of ferritin in HD patients. Finally prospective studies are needed to confirm whether elevated serum hepcidin and ferritin predict HD anemia and its elevation is failure execration by dialysis, or is simply a secondary marker of metabolic abnormalities.

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CONFLICT OF INTEREST

"No conflict of interest associated with this work".

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Table1: Demographic data of subjects included in the study

parameters	**Subjects**	
	patients	**controls**
Number of subjects	59	29
Males	37	17
Females	22	12
Age (years) Mean±SD;	28.3±6.4	25±3.5
Range of age (years)	20-45	20-40
BMI (Kg/m^2) Mean±SD	18.2±3.8	24.1±2.83

Table 2: Serum hepcidin and conventional markers of Iron status and markers of inflammation (CRP, WBCs) in hemodialysis patients and controls

Biochemical Parameters	**Patients (mean ± SD)**	**Controls (mean ± SD)**	***P* value**
Hb (g/dl)	7.97±1.4	15.55±1.3	<0.001
Serum Iron (mg/dL)	142.88±1	159.6±64	0.35
Serum Ferittin (ng/ml)	448.5±710	98.3±83	<0.001
CRP (mg/L)	21.3±28.6	2.9±2.7	<0.001
WBCs $x10^3$	5.6±1.7	5.0±1.16	0.10
Hepcidin (ng/ml)	63.77±47.4	11.5±26	<0.001

Hb=Hemoglobin
CRP=C-reactive protein
SD=Standard division

Table 3: Serum albumin and conventional markers of kidney function in hemodialysis patients and controls

Biochemical Parameters	**Patients (mean ± SD)**	**Controls (mean ± SD)**	***P***
Creatinine (mg/dl)	10.08±9.3	0.935±0.115	<0.001
Urea (mg/dL)	152.5±50.2	21.58±7.0	<0.001
BUN (ng/ml)	72.15±22.7	10.3±2.8	<0.001
Albumin (g/L)	34.7±4.5	43.15±2.47	<0.001

BUN =Blood urea nitrogen
SD=Standard division

Table 4: The association of high level of hepcidin with conventional markers of iron status and marker of inflammation (CRP) among hemodialysis patients

Biochemical parameters	High level of hepcidin n=37		*OR*	*CI*	χ^2	*P*
	No	%				
Serum Ferritin (high level) n=22	12	54.5	0.5	0.19-1.7	1.1	0.31
Iron µg/dl (Low level) n=8	7	87.5	4.9	0.5-42	2.5	0.11
Hb (g/dl) anemic n=58	36	62%	undefined		0.6	0.4
Serum CPR (Positive) n=31	20	64.5	1.2	0.4-3.3	0.09	0.76
Total high rate of Hepcidin n=59	37	62.7				

Serum Ferritin (high level); for male >275ng/ml; for female >204ng/ml.
Serum Iron (low level); for male <59 mg/dL; for female < 37 mg/dL.
Serum CPR Positive (>6mg/dL)
OR Odds ratio = Relative risk *CI* Confidence intervals χ^2 Chi-square = 3.9 or more significant *p* Probability value = 0.05 or less significant

Table 5: The associated odds ratio of low level of serum iron, high level of serum hepcidin, high level of serum ferritin, and positive CRP, in hemodialysis patients comparing with healthy controls.

Biochemical parameters	**Patients n=59**		**Controls n=29**		***OR***	***CI***	χ^2	***P***	**Risk difference**
	No	**%**	**No**	**%**					
Serum Ferritin (high level)	22	37.3	4	13.7	3.7	1.5-12	5.2	0.02	36(25-46)
Iron µg/dl (Low level)	8	13.6	0	0	1.5	1.3-1.8	4.3	0.037	36(25-46)
Serum CPR (Positive)	31	52.5	1	3.4	31	3.9-243	20.2	<0.001	46(32-61)
High level of hepcidin	37	62.7	2	6.9	22.7	4.9-104	24.5	<0.001	49(34-65)

OR Odds ratio = Relative risk ***CI*** Confidence intervals χ^2 Chi-square = 3.9 or more significant
p Probability value = 0.05 or less significant

Table 6: Comparison of high (> 6mg/l) and low (<6 mg/l) CRP levels in relation to age, gender and hemodialysis duration for patients.

Parameters	**High CPR (>6mg/L)**	**Low CRP (<6mg/L)**	***P***
Age (mean ± SD)	33.4±5.8 years	26.5±7.1years	<0.001
Gender			
Male	23	14	<0.05
Female	8	14	0.15
Total	31	28	0.84
Hemodialysis duration mean ± SD	4.9±0.6 years	3.8±0.4 years	0.08