



## SERUM HEPcidIN LEVELS IN ANEMIA OF CHRONIC KIDNEY DISEASES COMPARED TO IRON DEFICIENCY ANEMIA AND IT'S CORRELATION WITH SERUM LEVELS OF HS –C REACTIVE PROTEIN, INTERLUKIN-6 AND FERRITIN

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### ABSTRACT

Hepcidin, is a recently discovered small peptide secreted by the liver to act as a key regulator of iron homeostasis. Patients on hemodialysis(HD) are usually anemic ,because of defective erythropoiesis. The present study was directed to investigate serum levels of hepcidin in two types of anemia (anemia of chronic kidney diseases(HD) and iron deficiency anemia), Analyze the association between hepcidin levels and related inflammatory markers(IL6 and hs- CRP) together with hematological parameter ,to evaluate the role of hepcidin in differentiating specific types of anemia. Sixty-four female subjects aged 30.3±1.17 (means ± SEM) were participated in this study and divided into: Twenty patients with chronic kidney disease treated by hemodialysis ( creatinine clearance < 10 ml/min ,receiving recombinant human erythropoietin-alpha plus intravenous iron),twenty-one, newly diagnosed iron deficiency anemic patients(IDA), all of patients were having Hb < 12g/dl , and twenty- three apparently healthy subjects served as a control group. Serum hepcidin levels were statistically different, from high to low: HD > control >IDA. Serum ferritin levels were significantly increased in HD patients ,whereas decreased levels were detected in IDA. A positive correlation between serum hepcidin and IL-6 levels only existed in HD groups .Furthermore, serum hepcidin levels lost it's usual association with serum ferritin levels in (HD), as compared IDA and controls( $r=0.02$   $p>0.05$ ,  $r=0.76$   $p<0.001$ ,  $r=0.63$   $p<0.001$  ,respectively).Data demonstrated that serum hepcidin might play important role in pathogenesis of anemia of chronic kidney disease and IDA, but it's levels are affected by the associated inflammatory state ,which may be utilized as a biomarker for differentiation of these types of anemias.

**KEYWORDS:** Hepcidin, iron deficiency anemia, anemia of chronic diseases, hemodialysis, IL-6, hs-CRP , Ferritin.

### INTRODUCTION

Anemia occurs when there is a reduction in one or more of the major red blood cell measurements; hemoglobin concentration, hematocrit, or red blood cell count. <sup>(1)</sup> If it is due to transient blood loss or hemolysis while kidneys and bone marrow are normal, as the erythropoietic system acts to correct the anemia <sup>(2)</sup> .World Health Organization (WHO) defines anemia as hemoglobin levels less than 12.0g/dl in pre-menopausal women <sup>(3)</sup> .Etiology of anemia is frequently difficult to determine even after extensive investigations including bone marrow examinations. It is reported as nutritional (34%), renal insufficiency(12%), chronic diseases (20%)and unexplained(24%) <sup>(4)</sup> .

A normocytic normochromic anemia usually accompanies progressive of anemia of chronic kidney Disease (CKD)(5), and the overall prevalence of CKD-associated anemia is approximately 50%(6). Although anemia may be diagnosed in patients at any stage of CKD, there is a strong correlation between the prevalence of anemia and the severity of CKD.(staging of chronic kidney disease table-1) . Inadequate production of erythropoietin is commonly thought to be the most important factor in the pathogenesis of anemia in those patients, and many patient are treated with erythropoietin stimulating agents (ESAs). However, approximately 10-20% of patients are poorly responsive to ESAs(7).The etiology of anemia in

CKD is multifactorial(8), In addition to relative erythropoietin deficiency, shortened erythrocyte survival(9),and the erythropoiesis inhibitory effects of accumulating uremic toxins could also contribute to the anemia of CKD. Importantly, CKD patients may have several abnormalities in systemic homeostasis of iron, (an essential component )in the production of red blood cells(8). First, hemodialysis patients in particular are typically in negative iron balance, losing approximately 1-3 grams of iron per year, due in part to blood trapping in the dialysis apparatus and repeated phlebotomy. Second, many patients are on ESAs to manage their anemia, which depletes iron stores by driving increased production of red blood cells(9). Third, it has been recognized that CKD patients also have impaired absorption of dietary iron (10).

Recent studies suggest that the impaired intestinal iron absorption and impaired release of iron from body stores in CKD patients, like in other patients with anemia of inflammation, may be caused by an excess of the key iron regulatory hormone hepcidin<sup>(11,12)</sup>.Hepcidin, a small peptide produced by the liver, is a recently discovered central mediator of iron homeostasis<sup>(13)</sup>. Via regulation of ferroportin, hepcidin inhibits intestinal iron absorption and iron release from macrophages and hepatocytes. Because of its renal elimination and regulation by inflammation, it

is possible that progressive renal insufficiency leads to altered hepcidin metabolism, subsequently affecting enteric absorption of iron and the availability of iron stores. Thus, hepcidin likely plays a major role in the anemia of CKD as well as ESA resistance<sup>(14)</sup>. Hepcidin expression is reduced when erythropoiesis is iron restricted (iron deficiency anemia) but it is stimulated by inflammation<sup>(2)</sup>. In IDA, the iron supply depends on the amount of the iron stores, whereas in ACD, the supply depends on its rate of mobilization. In ACD, functional ID may occur even in the presence of large iron stores when iron release is impaired<sup>(15)</sup>. Recent studies indicate that pro-inflammatory cytokines play an important role in the pathophysiology of ACD by increasing iron accumulation and storage by monocytes /macrophages via hepcidin-dependent pathways<sup>(16)</sup>. Since hepcidin is directly implicated in the regulation of iron homeostasis,

in contrast to increased levels of hepcidin in ACD, both classic iron deficiency and iron deficiency anemia (IDA) are associated with low hepcidin levels<sup>(17,18)</sup>. Such correlation makes hepcidin a potential marker for different types of anemia.

In this article, we measured serum hepcidin in patients with anemia of CKD ( patients with chronic kidney disease-stage 5 and were on HD -as an example on ACD) , and IDA in order to:

- To evaluate the role of hepcidin as a marker of anemia in IDA and ACD and to compare its levels in two types of anemia.
- To evaluate correlation between serum levels of hepcidin and some markers of anemia and inflammation in both IDA and ACD.

**TABLE-1:** The National Service Framework for Renal Services Classification of CKD <sup>(3)</sup>

Stage	Features
Stage-1	Normal e GFR $\geq$ 90ml/min per 1.73m <sup>2</sup>
Stage-2	eGFR: 60 to 89 ml/min per 1.73 m <sup>2</sup>
Stage-3	eGFR:30-59 ml/min per 1.73 m <sup>2</sup>
Stage-4	eGFR:15-29 ml/min per 1.73 m <sup>2</sup>
Stage-5	eGFR:<15 ml/min per 1.73 m <sup>2</sup> or end stage renal disease
eGFR= estimated Glomerular Filtration Rate	

## SUBJECTS, MATERIALS AND METHODS

### Subjects

This study included twenty female patients with end-stage renal failure were treated by means of chronic hemodialysis-HD under supervision of a specialized physician, ( creatinine clearance < 10 ml/min) from Hemodialysis unit /Al-Diwaniya Hospital and Al Hakeem Teaching Hospital/Al –Najef city. Those patients required a regular hemodialysis for 3 hr a day 2-3 times per week, and were treated with recombinant human erythropoietin alpha(4,000IU), and intravenous iron(iron dextran100mg) weekly, in order to maintain Hb at recommended level (11.0-12.0g/dl) ,from those attending hospital from 2012 . In addition to twenty- one female patients with iron-deficiency anemia ,attending The National Center of Hematological Research / Baghdad . All patients were selected under supervision of a senior physician ,having Hb values <12g/dl. As control group twenty three apparently healthy age and sex matched subjects were selected also. Subjects characteristics (patients and control groups) are summarized in table -2 .Blood specimens were obtained before patient started hemodialysis. , by excluding the followings : - Hepatitis, Diabetes Mellitus. While, for iron deficiency anemic patients (IDA) included:- Anemic patients (Hb < 12 g/dl),- Non pregnant, Without any chronic disease.

### Specimens Collection and Evaluation

Whole blood samples were obtained using EDTA-tubes for measuring hematological markers: complete blood count , blood film , hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Hematological analysis was performed by Cell-DYN Ruby® Hematology analyzer (Abbott Diagnostics, USA). Blood smear was performed by using Atlantes® microscope on HP to exclude other type of anemia depending on the appearance of blood cells. Furthermore, serum was separated and kept frozen until analysis .Serum levels of hepcidin <sup>(19)</sup> , IL-6<sup>(20)</sup> were measured using commercially available ELISA kits (Cusabio Biotech, China) . Demeditec Diagnostics (Germany), purchased kits for determination of hs-CRP<sup>(21)</sup> Whilst ferritin <sup>(22)</sup> ELISA-Kit (Monobind Inc, USA) was also used to measure serum ferritin.

### Statistical analysis

SPSS, version 19; was used for data analysis. Values were expressed as means  $\pm$  SEM. One-way ANOVA was used to test for significant difference among studied groups. Multiple comparison analysis by least significant difference (LSD) was used. Correlation between variables was assessed by Pearson's correlation coefficient with P<0.05 was considered as statistically significant.

**TABLE-2** : Baseline Characteristic Of All The Groups

Variable	HD(n=20)	IDA (n=21)	Controls(n=23)
Age (years)	31.3±1.41	31.76±1.71	27.95±1.41
LBW (Kg)	49.43±0.89	51.73±0.56	51.22±0.6
BMI (Kg/m <sup>2</sup> )	22.34±0.89	25.3±1.18	23.90±0.68
HD Duration (month)	32.95±6.01	-	-
Ccr (ml/min)	8.6±0.44 <sup>▲</sup>	95.1±3.06	95.81±3.88
Hb (g/dl)	8.78±0.43 <sup>▲</sup>	9.85±0.38 <sup>x,▲</sup>	12.84±0.13
MCV (fl)	87.03±1.47	74.23±2.8 <sup>x,▲</sup>	86.85±0.97
MCH (pg)	29.06±0.7	23.2±1.1 <sup>x,▲</sup>	28.70±1.4
MCHC (g/dl)	32.88±0.49	29.8±0.41 <sup>x,▲</sup>	32.74±0.29

HD=hemodialysis, IDA=iron deficiency anemia, LBW = Lean body weight, BMI =Body mass index, Ccr=Creatinine clearance, Hb= Hemoglobin, MCV= Mean corpuscular volume, MCH =Mean corpuscular hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, Creatinine Clearance was calculated by Cockcroft-Gault equation [ $CCr = 0.85 * (140 - age)LBW / (Plasma Cr * 72)$ ]. where, LBW=Lean Body Weight (Kg).

## RESULTS

Concentration of Hb in IDA patients was significantly lower than that of HD patients ( $p < 0.001$ ) as shown in table- 1. Mean values of MCV and MCH, indicated that patients on hemodialysis had normocytic - normochromic type of anemia, however, these were significantly different from both controls and IDA patients, but the latter presented with microcytic - hypochromic anemia (MCV=74.23fl and MCH=23.2pg, respectively). A significant reduction in MCHC in patients with IDA compared to both HD and control subjects (table-1). In this study serum hepcidin levels in anemics on hemodialysis (326.02ng/ml) was significantly higher than that of iron deficiency anemic patients (6.21ng/ml,  $P < 0.001$ ) and controls (70.12ng/ml,  $P < 0.001$ ). Meanwhile, hepcidin levels in IDA was significantly lowered even than that of control ( $P = 0.01$ ), as illustrated

in figure-1 a .In fact, serum hepcidin values in the studied groups can be ordered from high to low: HD > control > IDA. HD patients had significantly higher levels of serum ferritin (532.12ng/ml) when compared with IDA patients (8.29ng/ml,  $P < 0.001$ ) and controls (22.73ng/ml,  $P < 0.001$ ) as seen in figure1-b. Furthermore, Increased serum IL-6 levels were detected in HD (9.19 pg/ml,  $P < 0.001$ ) but not in patients with IDA (1.63 pg/ml,  $P > 0.05$ ) compared to controls, (figure-1 c). Similarly, serum hs-CRP levels were (7.59 µg/ml), higher than that of IDA and controls (1.02µg/ml and 1.09 µg/ml, respectively) with ( $P < 0.001$ ) figure-1 d ).

## Correlations Studies

The association between studied variables had been tested by Pearson's correlation coefficient. with P-values < 0.05 was considered as statistically significant ( table-3).

**TABLE 3:** Correlations of Hepcidin Levels with Biochemical and Clinical Variables in Studied Groups

Variable	HD		IDA		Controls	
	R	p-value	R	p-value	R	p-value
Age (years)	-0.06	Ns	0.02	Ns	0.17	Ns
LBW (Kg)	0.58*	0.02	0.05	Ns	0.27	Ns
BMI (Kg/m <sup>2</sup> )	-0.33	Ns	-0.07	Ns	-0.07	Ns
HD Duration(month)	-0.4	Ns	-	-	-	-
Ccr (ml/min)	0.12	Ns	0.03	Ns	0.2	Ns
Hb (g/dl)	0.27	Ns	0.41	Ns	0.25	Ns
MCV (fl)	0.05	Ns	0.25	Ns	-0.24	Ns
MCH (pg)	0.13	Ns	0.14	Ns	-0.28	Ns
MCHC (g/dl)	0.14	Ns	0.08	Ns	0.43*	0.04
Ferritin (ng/ml)	0.02	Ns	0.76**	<0.001	0.63**	0.001
IL-6 (pg/ml)	0.76**	0.001	0.04	Ns	0.17	Ns
CRP (µg/ml)	0.34	Ns	0.01	Ns	-0.13	Ns

HD=hemodialysis, IDA=iron deficiency anemia, LBW = Lean body weight, BMI =Body mass index, Ccr=Creatinine clearance, Hb= Hemoglobin, MCV= Mean corpuscular volume, MCH =Mean corpuscular hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, IL-6=interleukin-6, CRP=C-reactive protein, \*\* =  $p \leq 0.01$ , \* =  $p \leq 0.05$ .

## Hepcidin and iron parameters

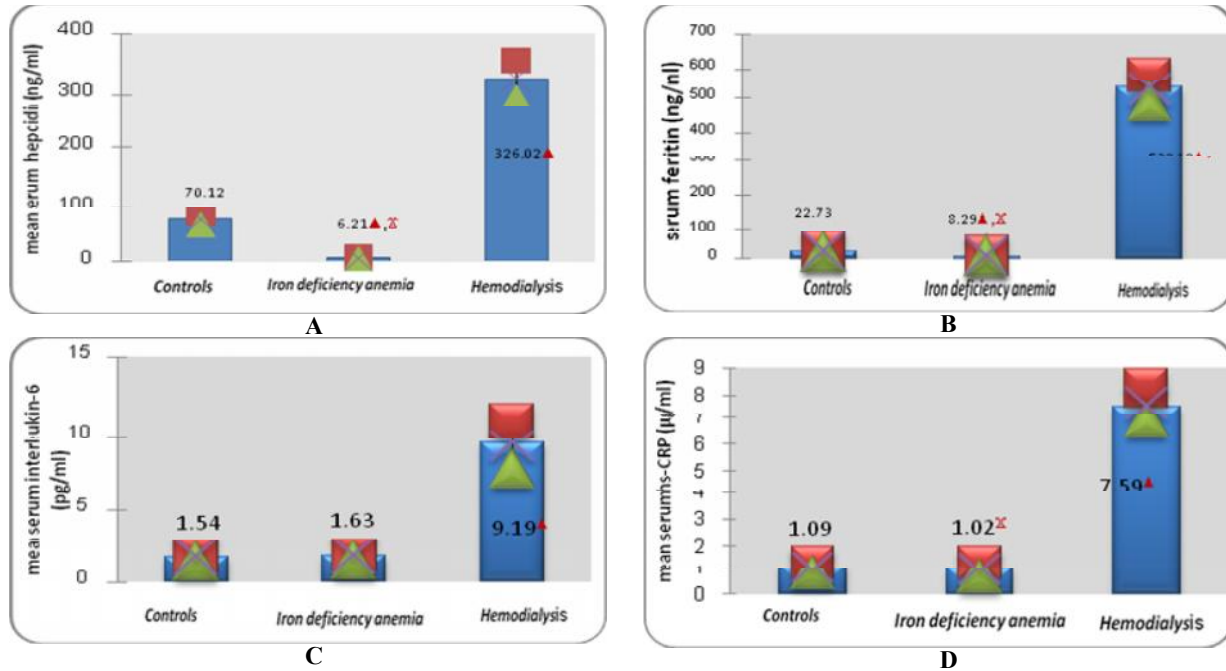
By investigating the correlation between serum hepcidin and tested hematological parameters, we found that no correlations were detected between serum hepcidin and

Hb, MCV, MCH in our study. However, there was a significant positive association between serum hepcidin and serum ferritin levels in both control and IDA groups ( $r = 0.633$ ,  $p = 0.001$  and  $r = 0.758$ ,  $p < 0.001$ , respectively);

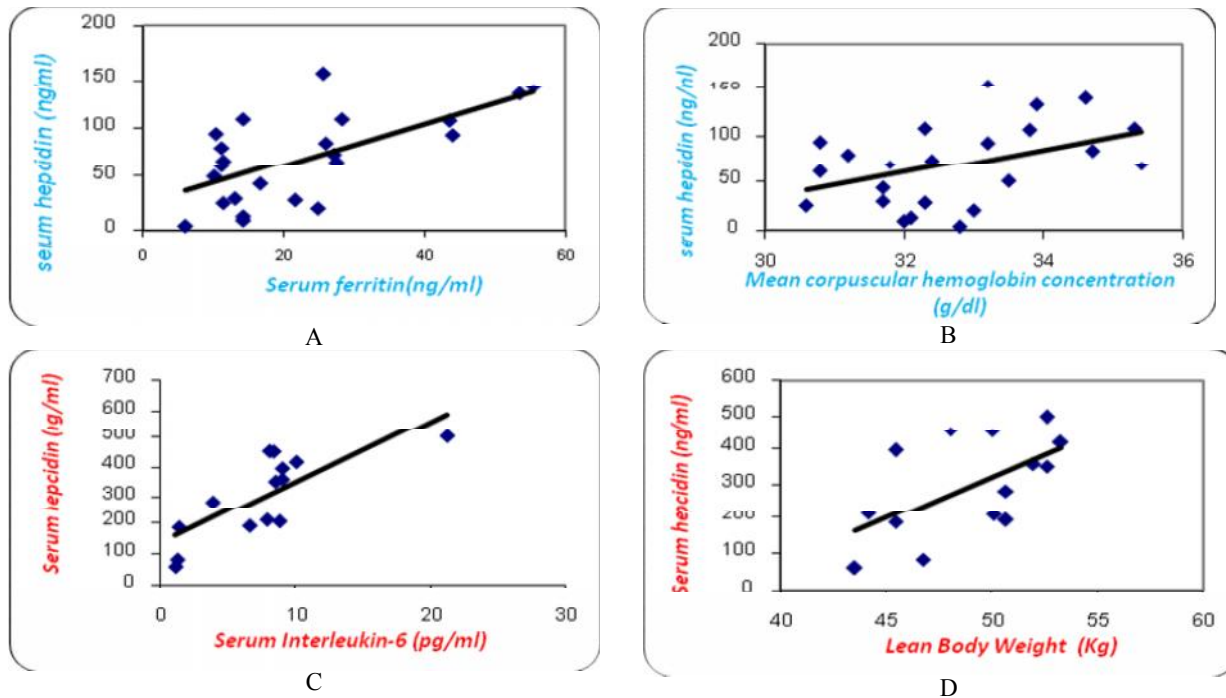
figure- 2 a and e ). Meanwhile serum hepcidin levels exert a significant positive correlation with MCHC values in control group ( $r = 0.430, p = 0.040$  at the  $p < 0.05$  level) figure-2 b.

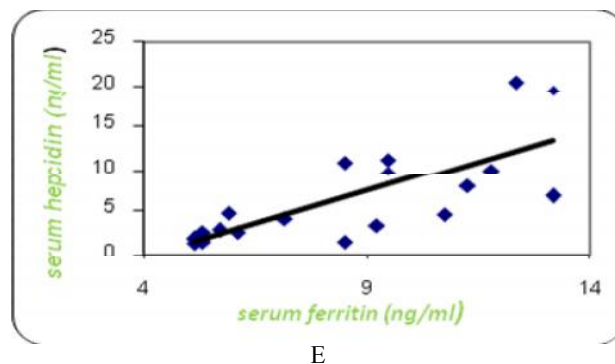
#### Hepcidin and Inflammatory Markers

Serum IL-6 level in HD group was significantly correlated with hepcidin level ( $r = 0.764, p = 0.001$ ), figure-2 c, but serum CRP level was not. While there were no correlations between inflammatory markers (IL-6 and and hepcidin in other groups.



(Figure- 1) Levels of Serum : (a) hepcidin, (b) ferritin, (C) IL-6, and (d) hs- CRP among groups. , IL-6=interleukin-6, CRP=C-reactive protein , the results are expressed as mean  $\pm$  SE, ▲ =significantly different from control ;  $p < 0.05$  , ×=significantly different from HD group ;  $p < 0.05$  .





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(Figure- 2) Pearson's Correlation between serum hepcidin and studied parameters : *a* -Correlation with serum ferritin in control . *b*- Correlation with mean corpuscular hemoglobin concentration in control. *C*- Correlation with serum interleukin-6 in patients on hemodialysis. *d*-Correlation with lean body weight in patients on hemodialysis. *e* - Correlation with serum ferritin in iron deficiency anemic patients.

## DISCUSSION

Some patients with chronic kidney disease (CKD) are reported to show dysregulation of iron metabolism and develop anemia of chronic disease (ACD)<sup>(23)</sup>. A hallmark of ACD is abnormal iron homeostasis associated with increased uptake and retention of iron by cells in the reticuloendothelial system. This might lead to diversion of iron from the circulation to storage sites within the reticuloendothelial system that limits the availability of iron for erythroid progenitor cells, and iron-restricted erythropoiesis<sup>(24)</sup>. The observation that polymorphonuclear cells from patients on maintenance hemodialysis had 2–3 times the iron content as cells of healthy subjects reflecting the defective regulation of iron transport proteins<sup>(25)</sup>.

In agreement with previous data<sup>(17,24)</sup>, The present study demonstrated that serum hepcidin levels were higher in HD patients than in control subjects and IDA patients, figure(1-a). As expected from studies in other populations, the elevation of serum hepcidin appears to be multifactorial, hepcidin production is regulated by iron, inflammation and erythropoiesis<sup>(17,26)</sup>. when we measured IL-6 and hs- CRP to examine whether elevated serum hepcidin may be ,at least, partly due to inflammatory signals presented as significant elevation in IL-6 and CRP levels were found in HD, figure(1-c,d). These data suggested that inflammatory cytokines were able to strongly stimulate hepcidin expression and such induction was responsible for the hypoferrremia that accompanied inflammatory episodes<sup>(17)</sup>. Inflammation is a well-known cause of erythropoietin stimulating agents resistance in HD patients. Hence,HD per se, without apparent infection, should be considered an inflammatory condition. The reason is not entirely clear, but the presence of endotoxins in the dialysis solution, blood contact with the dialysis membrane, and uremia per se could all be involved in the inflammatory response<sup>(27)</sup>. It is also explained as disturbance of acquired immunity in HD patients, due to impaired interaction between the antigen-presenting cell and T-lymphocyte<sup>(28)</sup>.Under chronic inflammatory conditions, excessive production of cytokines produced by macrophages and T-lymphocytes, and inflammatory cytokines particularly IL-6 plays a central role in hepcidin production<sup>(16)</sup> through induction and binding of signal transducer and activator of

transcription 3 (STAT 3) to hepcidin gene promoter<sup>(29)</sup>.In agreement with other studies<sup>(17,24)</sup>, we found that serum hepcidin levels strongly correlated with serum IL-6 levels, which would seem to support this proposed mechanism, figure(2-c). Nemeth et al. have shown that the cytokine IL-6 is necessary and sufficient for induction of hepcidin during inflammation, establishing that the iron regulatory peptide plays a key role in the anemia of chronic diseases<sup>(16)</sup>. In human hepatocyte culture hepcidin is induced by IL-6, but not IL-1 or TNF- $\alpha$ , indicating that hepcidin induction by inflammation , is a type II acute-phase response<sup>(15)</sup>. Besides, IL-6 played an important role in regulation of hepcidin in anemia of hemodialysis but not in IDA according to Pearson's correlation. Serum hs-CRP and ferritin concentrations were increased in hemodialysis as compared to controls and iron deficiency anemia levels,figure(1-b,d), but not correlated with serum hepcidin which is consistent with a previous study<sup>(27)</sup>.Kato et al, didn't found a significant association between serum CRP and hepcidin in patients on hemodialysis<sup>(30)</sup>. Several factors may explain such lack of relationship, including differences in the half-lives of hs-CRP and hepcidin<sup>(31)</sup>. Another explanation may be the distinct features of the studied population<sup>(32)</sup>. Elevated serum levels of ferritin in HD group could be attributed to that, serum ferritin is a potent acute phase reactant, and usually is associated with elevation of CRP levels and inflammation<sup>(33)</sup>. Inflammatory cytokines may block Iron from going to bone marrow for utilization or to increase the release of serum ferritin in the blood<sup>(34)</sup>. Production of hepcidin by inflammatory activators could cause disturbed release of iron from the reticuloendothelial system resulting in high ferritin levels and non utilization of the parenteral iron administered<sup>(35)</sup>. Recently Damien. et al. demonstrated that hepcidin levels were significantly elevated in HD patients but not correlated with ferritin<sup>(36)</sup>. While another study, reported a close correlation between hepcidin and ferritin<sup>(24)</sup>. The difference could be explained by the fact that ferritin levels of patients in the former study were very high as in our research. Although the association with ferritin was lost in the hemodialysis group. Target-driven therapy with supplemental intravenous iron to predefined the goal ferritin levels may have confounded the relationship in hemodialysis group<sup>(14)</sup>.Meanwhile , there was no correlation between serum ferritin and CRP levels

this was counterintuitive, as ferritin was a well known acute phase reactant. The failure of utilization of serum ferritin to distinguish between IDA and ACD is not due only to the decrease in serum ferritin levels in IDA, but also to the increase in serum ferritin caused by the acute phase response associated with chronic inflammatory diseases as in ACD<sup>(2)</sup>.

Serum hepcidin levels in IDA were significantly lower than those in HD and the control as shown in table-1, which was coordinate with results from other studies because serum prohepcidin levels were significantly lowered in IDA patients compared with both control and ACD subjects<sup>(17,37)</sup>. However, there was a significant positive correlation between serum hepcidin and ferritin in both IDA and controls, figure -2 a,e. This is consistent with numerous studies that have documented a positive correlation between ferritin and hepcidin<sup>(4,38,39)</sup>. Serum ferritin was shown to be the most important marker correlates with serum hepcidin concentration. A positive regression coefficient was found, indicating that increased serum ferritin concentration is associated with increased serum hepcidin concentration, which has consistently been reported before<sup>(39)</sup>. Because hepcidin shifts the iron from the functional compartment to the storage one, part of which is ferritin. In addition ferritin could be the stimulus for hepcidin synthesis<sup>(40)</sup>. In healthy subjects, hepatic hepcidin production is regulated by a feedback mechanism induced by circulating iron. Low levels of circulating hepcidin allow ferroportin to release iron into the bloodstream; however, elevated levels of hepcidin effectively reduce iron absorption in enterocytes by disabling the iron exporter ferroportin<sup>(41)</sup>. Apparently, this mechanism observed in ferroportin present in enterocytes functions differently from ferroportin of macrophages or hepatocytes. Dorine W. et al., as with our results, reported that, serum hepcidin levels correlated significantly with ferritin in control and IDA. These findings are in agreement with previous findings on the increase of human hepcidin synthesis by iron stores and thereby demonstrate the usefulness of the hepcidin assay in clinical studies<sup>(42)</sup>. Anemia in HD patients is a multifactorial condition and its clinical management remains challenging. The interactions between iron metabolism, erythropoietin deficiency and chronic inflammation are difficult to dissect and new markers are urgently needed to optimize treatment approaches<sup>(17)</sup>. It is now widely accepted that hepcidin is the key regulator of overall body iron homeostasis. Indeed, several regulators of iron homeostasis were recognized to control iron- flux in response to reductions in tissue oxygen levels, changes in body iron stores, alterations in erythropoiesis, inflammation and infection. Recent findings demonstrated that hepcidin gene expression is regulated by all these physiological changes, thus providing evidence that it is the long-sought iron regulatory hormone<sup>(43)</sup>. In conclusion, serum hepcidin levels increased in HD and decreased in IDA, with significant difference between the two types of anemias. Thus hepcidin might be a good tool in discrimination of the type of anemia. Although serum hepcidin changes parallel to those observed with serum ferritin (but with a greater magnitude for hepcidin), however, the observation of elevated serum inflammatory

markers(IL-6 and CRP) in one of the two types of anemia (HD), indicating the role of inflammation in HD patients, but not in IDA, with high elevation in serum levels of hepcidin and ferritin in those patients, as acute phase reactant proteins, but lowered levels for hepcidin was not related to inflammatory state in IDA patients, as presented by non significant alteration in serum level of IL6 and CRP. We emphasize the importance of additional studies that do allow elucidation of causality between hepcidin, ferritin and other hematological variables in different types of anemia.

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