Correlation between Anemia and Hepcidin-25 and Interleukin 6 Levels in Early Stage and Advanced Chronic Renal Disease

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ABSTRACT

Background: Anemia is very commonly seen complication in the patients with chronic renal disease (CRD). This is thought to result from decreased renal excretion of the hepcidin and chronic inflammatory process. Our study aimed to investigate the correlation between hemoglobin (Hb) and hepcidin levels in all stages starting from stage 2 CRD.

Method: The study was conducted on a total of 80 patients with stage 2-3-4-5 chronic renal disease who have not been treated with renal replacement therapy, including 20 patients from each stage. In all groups, C-reactive protein (CRP), high sensitivity C-reactive protein (HsCRP), interleukin 6 (IL 6), hepcidin-25, routine biochemistry, whole blood count, iron, total iron binding capacity (TIBC), ferritin, folic acid and Vitamin B12 were measured.

Results: When the patients were evaluated for inflammatory parameters, CRP (p=0.01), HsCRP (p=0.01), erythrocyte sedimentation rate (ESR) (p=0.001), IL 6 (p=0.001), ferritin (p=0.02), hepcidin-25 (p=0.03) levels were significantly higher in the group with advanced stage compared to the group with early stage. It was determined that hemoglobin (Hb) level was statistically significantly lower in advanced CRD compared to early stage CRD ($p<0.001$).

Conclusion: Using the agents that may decrease the levels of hepcidin may be useful to increase Hb levels in the treatment of anemia in the patients with chronic renal disease.

Keywords: Anaemia, chronic renal failure, hepcidin

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INTRODUCTION

Chronic renal disease (CRD) is a pathophysiological process with several etiological factors, which results to progressive and irreversible decrease of nephron count and functions and commonly progresses to end-stage renal disease (1).

Many patients with chronic renal disease and anemia may be effectively treated using erythropoiesis-stimulating agents (ESA). Nevertheless, approximately 10% of the patients are slightly sensitive or insensitive to such agents (2-4). When such patients were examined, it was found that the resistance was due to functional iron deficiency.

Hepcidin is very newly discovered, important protein with low molecular weight that plays a role in the iron metabolism and it is excreted via urine (7). It serves as a type 2 acute phase reactant synthesized in the liver (6-8). It is thought to play a key role for systemic homeostasis of the iron. While high levels of iron and inflammation increase the production of hepcidin, the synthesis of hepcidin decreases with iron deficiency, increased erythropoiesis, hypoxia, anemia and ESA (9).

In the studies performed, it was found that the levels of hepcidin, ferritin, IL-6 and other acute phase reactants were increased and well inter-correlated in the patients with chronic renal disease (5).

When viewed from this aspect, although it is thought that hepcidin has an important place in chronic renal disease, the correlation between the stages of CRD, anemia and hepcidin levels has not been clearly revealed yet. Therefore, our study aimed to investigate the correlation between anemia and hepcidin levels in all stages starting from stage 2 CRD.

MATERIAL AND METHOD

The study included the patients with stage 2-3-4-5 chronic renal disease who have been admitted to Ministry of Health, Ankara Training and Research Hospital, Nephrology Clinic between September 2009 and April 2010 and who did not receive renal replacement therapy. A total of 80 patients were enrolled to the study, 20 patients from each stage. The patients were classified into two groups as early stage (GFR >30 ml/min) and advanced stage (GFR<30 ml/min).

The exclusion criteria were acute or chronic infection, history of inflammatory disease, history of malignancy, aplastic anemia, history of hemochromatosis, CRP level >5-fold, procalcitonin level >5 ng/ml and the use of erythropoietic stimulant agents.

As described in the Declaration of Helsinki, all patients were informed about the study and its procedures and they gave a written consent. After taking a detailed medical history of the patients, routine physical examination was performed. For all patients, height and weight were measured and body mass index (BMI) was calculated by dividing the weight expressed in kilogram to the square of the height expressed in meter. Following a 15-minute resting period, arterial blood pressure was measured from the right arm using mercury sphygmomanometer.

In all groups, blood samples were drawn between 09:00 AM and 11:00 AM following 9 to 16-hour fasting period. In the samples obtained, routine biochemistry, whole blood count, CRP, HsCRP, total iron, total iron binding capasity, ferritin, transferrin levels were analyzed at the same day in the hospital using autoanalyzers. In addition, 5 ml blood sample was separated to analyze hepcidin and IL-6 levels. These samples were centrifuged at 4000 rpm for 5 minutes and then stored at -80 ° C.

In the central biochemistry laboratory, glucose, total cholesterol, HDL cholesterol, triglyceride, urea, creatinine and uric acid levels were measured using enzymatic colorimetric method and

total protein, albumin and magnesium were measured using colorimetric method on Olympus AV 640 device. LDL cholesterol was calculated using Fridewald formula. HsCRP was measured using HPLC method on Beckman-Coulter device. Ferritin was analyzed using chemiluminescent immunoassay method on Beckman Coulter Unicel DXI 800 device.

Hepcidin-25 and IL-6 levels were evaluated using enzyme-linked immunosorbent assay. The results were expressed in ng/ml.

Glomerular filtration rate was calculated using Modification of Diet in Renal Disease (MDRD) formula.

Statistical analyses were performed with the SPSS 16.0 (SPSS, Chicago, Illinois, USA) statistical package for Windows. All parameters were investigated using Kolmogorov-Smirnov test to determine whether or not they are normally distributed. The correlation coefficients and their significance were calculated using Spearman or Pearson test according to normality of distribution. Comparisons of continuous variables were made using the Mann-Whitney U test and student T test depending on normality of distribution. Categorical variables were compared by the Pearson's or Fisher's exact Chi2 tests.

RESULTS

Study group included a total of 80 patients, consisted of 20 patients with stage 2 and 20 with stage 3 for early stage disease and of 20 patients with stage 4 and 20 with stage 5 for advanced stage disease. Demographics and clinical characteristics of the patients are given in Table 1. Two groups were not different in terms of age and gender. When etiological factors of the patients were examined, most important etiological factors were found to be diabetes and hypertension

both in the patients with early stage disease and those with advanced disease. No significant difference was found across BMIs of the patients. For clinical findings, systolic and diastolic arterial blood pressures were compared and no significant difference was found. (p=0.90, p=0.78)

Table 1. Demographics and clinical characteristics of the patients enrolled.

	Stage 2-3 n= 40 (%)	Stage 4-5 N= 40 (%)	P value
Gender (F/M)	40 (18/22)	40 (23/17)	p = 0.07
Age	54.2 ± 10.5	57.3± 12.6	p = 0.07
Etiology			p = 0.06
DM (isolated)	3 (%7.5)	6 (%15)	
HT (isolated)	10 (%22.5)	10 (%25)	
DM+HT	10 (%25)	10 (%25)	
Chronic GMN	4 (%10)	5 (%12.5)	
Polycystic K.D	2 (%5)	3 (%7.5)	
Other	11 (%30)	7 (% 15)	
BMI (kg/m²)	28.3±4.7	27.8±6.3	p = 0.60
Systolic TA	134±20.7	131.1±20	p = 0.90
Diastolic TA	81.5±10.9	80.6±12.9	p = 0.78

(F: Female, M: Male, DM: Diabetes Mellitus, HT: Hypertension, GMN: Glomerulonephritis, BMI: body mass index)

The biochemical parameters of the patients by stage are given in table 2.

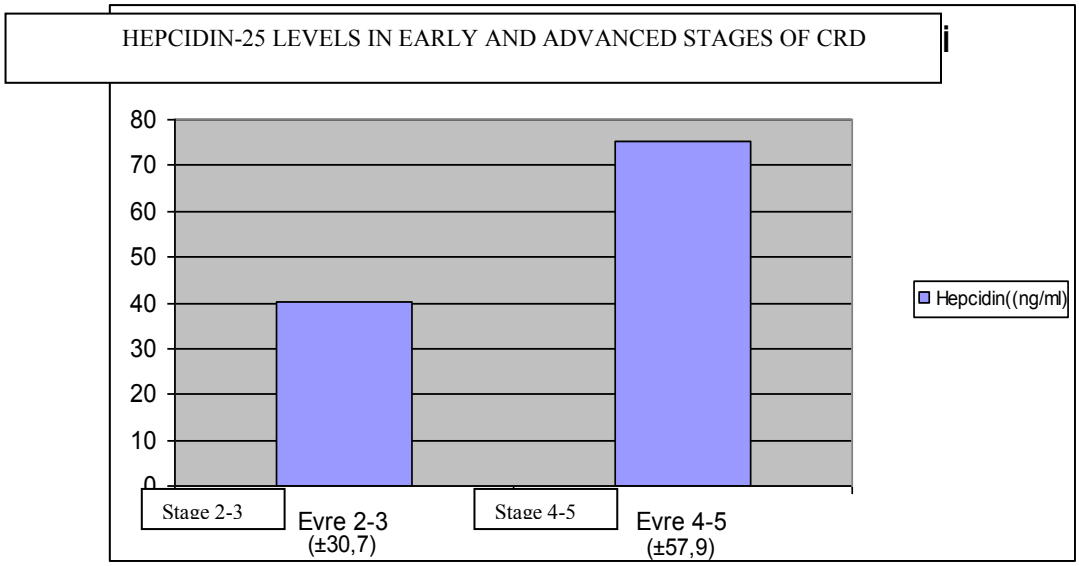
Table 2. Biochemical parameters of the patients by stage.

	Stage 2-3	**Stage 4-5**	**p value**
Hb (g/dl) (Mean ± SD)	13.6±1.8	10.9±1.9	**p< 0.001**
Htc (%) (Mean ± SD)	40.7±5.4	32.8±5.7	**p< 0.001**
Iron (ug/dl) (Mean ± SD)	70.1±22	49.9±20.9	**p< 0.001**
TDBK (ug/dl) (Mean ± SD)	333±53.4	270±92.2	**p< 0.001**
TSAT (%) (Mean ± SD)	22±6.8	23.6±22	p = 0.06
Ferritin (ng/ml) (Median (min-max))	57.8 (14-250)	123.1 (6-1045)	**p=0.02**
MDRD (Mean ± SD)	56.1±13.8	16.1±7.6	**p< 0.001**
Hepcidin-25 (ng/ml) (Mean ± SD)	40.3±30.7	75.3±57.9	**p=0.03**
CRP (mg/dl) (Median (min-max))	0.40 (0-1)	0.70 (0-7)	**p=0.01**
HsCRP (mg/l) (Median (min-max))	3.89 (0-14)	7.02 (0-32)	**p=0.01**
IL 6 (ng/ml) (Mean ± SD)	16.1±34	20.5±21.1	**p=0.001**
ESR (mm/hr) (Mean ± SD)	17.3±11.5	26.5±13.6	**p=0.001**
Vit. B12 (pg/ml) (Median (min-max))	273.5 (123-540)	298.5(102-2000)	p=0.37
Folate (ng/ml) (Median (min-max))	7.30 (4.25-24)	7.20 (2.01-24)	p=0.45
Urea (mg/dl) (Mean ± SD)	44.3±16.1	115.6±12.6	**p< 0.001**
Creatinine (mg/dl) (Median (min-max))	1.32 (1-2)	4.1 (2-15)	**p< 0.001**
Albumin (g/dl) (Median (min-max))	4.1 (3.6-4.9)	3.8(1-4.5)	**p< 0.001**
T. protein (g/dl) (Median (min-max))	7.4 (5.7-9.0)	7.1 (3.5-9.0)	p=0.20
T. cholesterol (mg/dl) (Mean ± SD)	202.2±44.7	187.4±56.2	p=0.13
HDL cholesterol (mg/dl) (Median (min-	42.5 (26-100)	40 (23-68)	p=0.49
LDL cholesterol (mg/dl) (Mean ± SD)	117.7±32.1	112.3±44.3	p=0.25
Triglyceride (mg/dl) (Median (min-	196.5 (53-913)	139.5 (45-543)	**p=0.01**
PTH (pg/ml) (Median (min-max))	61.5 (23-186)	2.5(34-821)	**p< 0.001**
Ca (mg/dl) (Mean ± SD)	9.5±0.4	8.5±0.8	**p< 0.001**
P (mg/dl) (Mean ± SD)	3.2±0.6	4.2±1.1	**p< 0.001**
Total protein (24h urine) (gr/day)	692.3±968	1934.5±2382	p= 0.60

In our study, when we examined the Hb and Htc values, statistically significant difference was detected between the patients with early stage and those with advanced disease. In the patients with advanced disease, Hb ($p<0.001$) and Htc ($p<0.001$) values were significantly lower compared to those with early stage disease (Table-2).

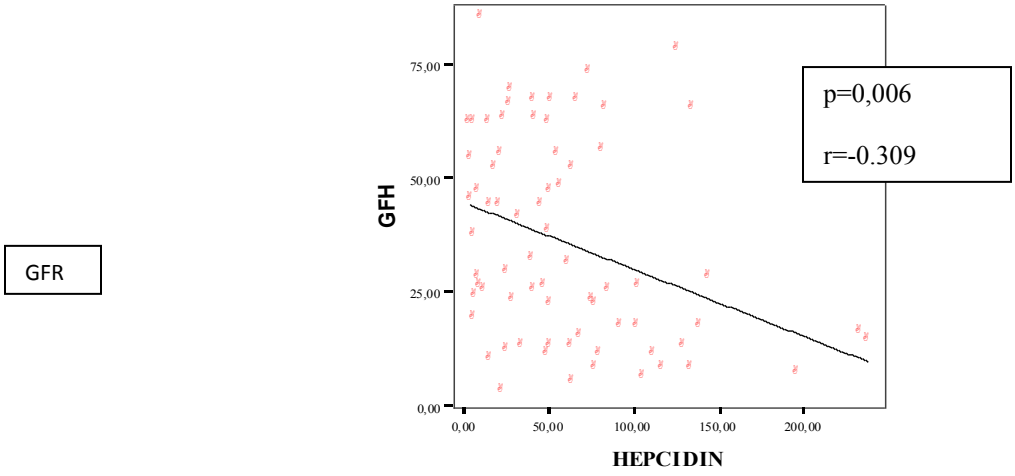
When the patients were evaluated for inflammatory parameters, CRP, HsCRP, ESR, IL 6 and ferritin levels were significantly lower in the patients with early stage compared to those with advanced disease ($p=0.01$; $p=0.01$; $p=0.001$; $p=0.001$; $p=0.02$, respectively). In addition, albumin, which has a negative acute phase reactant property, was found to be significantly lower in the patients with advanced disease compared to the patients with early stage disease ($p< 0.001$).

Hepcidin-25 levels were compared between the groups and a statistically significant difference was found between the patients with early stage disease and those with advanced disease ($p=0.03$). Accordingly, it was found that hepcidin-25 levels were significantly higher in the patients with advanced disease compared to those with early stage disease ($p=0.03$). Hepcidin-25 levels in early and advanced stages is seen in Graphic 1.



Graphic 1. Hepcidin-25 levels in early and advanced stages.

When all patients with CRD were evaluated (n=80), statistically significant negative correlation was found between GFR and hepcidin-25 level (p=0.006, r=-0.309). However, statistical significance of this correlation disappeared when the patients were classified in early stage (n=40) and advanced stage (n=40) groups (r = -0.224; p=0.17); r = -0.246; p=0.12, respectively). Correlation between GFR and hepcidin-25 is seen in Graphic 2.



Graphic 2. Correlation between GFR and hepcidin-25 levels in all patients with CRD.

In the patients with stage 2-3 disease, hepcidin-25 levels were examined using correlation analysis. A significant positive correlation was found between serum ferritin level and hepcidin-25 in the group with early stage. ($r = 0.759$; $p< 0.001$). However, no correlation was found between hepcidin-25 and Hb ($r = 0.027$; $p=0.86$), Htc ($r =0.018$; $p=0.90$), CRP($r = -0.183$; $p=0.26$), Hs CRP ($r = 0.112$; $p=0.49$), IL 6 ($r = 0.162$; $p> 0.32$) , ESR ($r = - 0.119$; $p=0.47$), iron saturation ($r = 0.262$; $p=0.10$), albumin ($r = 0.116$; $p=0.48$) and other parameters in the early stage.

In the correlation analysis of the hepcidin-25 levels in the patients with stage 4-5, these patients showed a significant positive correlation between hepcidin-25 levels and serum ferritin ($r = 0.796$; $p< 0.001$) and IL 6 ($r =0.576$; $p< 0.001$) levels. In addition, a significant negative correlation was found between hepcidin levels and Hb ($r = -0.325$; $p=0.04$) , Htc ($r = -0.305$; $p=0.05$), parathormone ($r = -0.318$; $p=0.04$) values of the patients. No significant correlation was found with any other parameter.

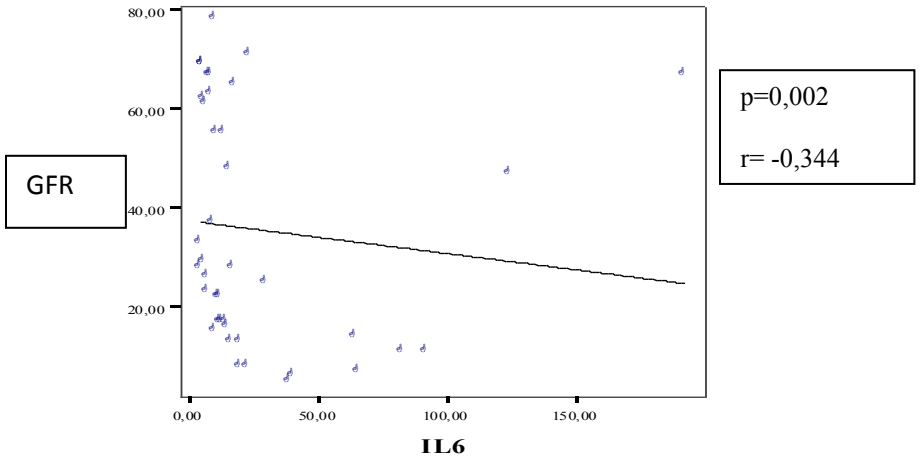
In the patients with stage 2-3 disease, GFR values were examined using correlation analysis. A positive correlation was found between IL-6 ($r = 0.338$; $p=0.03$) and GFR in the group with early stage. No correlation was found with any other parameter.

In the correlation analysis of the GFR values in the patients with stage 4-5, a negative correlation was found between IL-6 ($r = -0.441$; $p=0.005$) and GFR and a positive correlation was found between GFR and Hb ($r = 0.524$; $p= 0.01$) and Htc ($r = 0.513$; $p= 0.01$).

In the patients with early stage disease, IL-6 levels were examined using correlation analysis. A positive correlation was found between CRP ($r = 0,417$; $p= 0.007$), GFH ($r = 0,338$; $p=0.03$), HsCRP ($r = 0,448$; $p< 0.004$) and IL 6 levels.

In the patients with advanced stage disease, a positive correlation was found between IL-6 level and ferritin (r = 0.428; p=0.007) and hepcidin-25 (r = 0.576; p< 0.001) and a negative correlation across GFR (r = -0.441; p= 0.005), Hb (r = -0.415; p=0.009) and Htc (r = -0.384; p=0.01) values.

When all patient groups were evaluated (n=80), a statistically significant negative correlation was detected between IL-6 and GFR (p=0.002, r = - 0.344). The correlation between IL-6 and GFR was shown in Graphic 3.



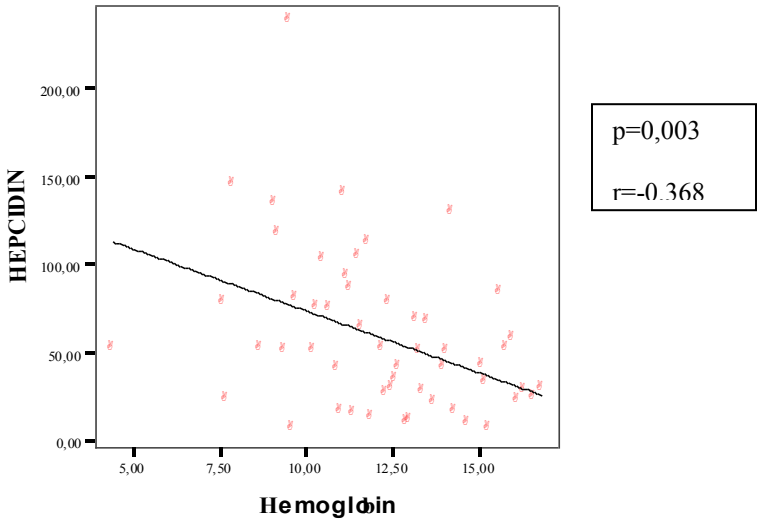
Graphic 3. Correlation between IL-6 and GFR in all patients with CRD

In the patients with Stage 2-3, Hb and Htc levels did not show a correlation with any parameter.

In the patients with Stage 4-5, a positive correlation was detected between Hb and total protein (r = 0.586; p< 0.001), between Hb and albumin (r =0.546; p< 0,001), between Hb and Ca (r = 0,344, p=0.03) and between Hb and GFR (r = 0.524; p = 0.001). In addition, statistically

significant negative correlation was observed between Hb and phosphorus (r = -0.605; p< 0.001), Hb and hepcidin (r = -0.325; p=0.04) and Hb and IL 6 (r = - 0.415; p=0.009).

In the entire CRD group, statistically significant negative correlation was found between Hb and hepcidin-25 (p= 0.003, r = - 0.368). Graphic 4 showed the correlation between Hb and hepcidin-25 in all CRD group.



Graphic 4. Correlation between Hb and hepcidin levels.

DISCUSSION

Anemia is one of the leading complications of the chronic renal disease. It is one of the important mortality and morbidity factor and it becomes deeper during the course of the disease, in parallel to loss of renal functions. Although a significant progress has been made in the treatment of anemia in CRD upon the introduction of the erythropoietin therapy in the clinical practice, it

remains to be an important problem (10). Hepcidin is a peptide hormone that was discovered in the last years and serves as a homeostatic regulator of intestinal iron absorption, iron cycle in the macrophages and iron release from hepatic stocks. At the same time, it serves as a type 2 acute phase reactant synthesized in the liver (11).

In our study, we aimed to reveal the correlation between anemia, hepcidin and other inflammatory markers in chronic renal disease.

In NHANES study that included more than 15.000 subjects, it was demonstrated that the prevalence of anemia (Hb<12 gr/dl in men, Hb<11 gr/dl in women) was 1% when GFH was equal to 60 ml/min/1.73 m2, increased to 9% when GFH decreased to 30 ml/min/1.73 m2 and increased to 33-67% when GFH was 15 ml/min/1.73 m2 (12).

Hb levels begin to decrease starting from early stages of CRD and reach lower levels with advanced renal dysfunction. It was shown that each decrease by 10 ml/min/1.73 m2 in the glomerular filtration rate caused a decrease by 3% in the hematocrit (13).

In our study, the patients with early stage disease and those with advanced stage disease were compared and significant difference was found between two groups in terms of Hb levels and the incidence of anemia (**p< 0.001, p< 0.001,** respectively), consistently with previous studies that showed a correlation between anemia and decreased GFR in the patients with CRD.

For the anemia observed in CRD, second leading causal factor is iron deficiency. KDOQI recommends that serum ferritin level is maintained above 100 ng/ml in the patients with CRD(14).

In our study, when the patients were examined for their ferritin levels, it was found that ferritin levels were statistically significantly higher in the group with advanced disease compared to that with early stage disease (**p=0.03).**

It is though that the current situation is a functional iron deficiency rather than a simple iron deficiency and is basically associated with chronic systemic inflammation. However, data are still limited to explain the pathophysiology of this condition.

Chronic systemic inflammation is very diffused, even generalized, component in the patients with chronic renal disease (15). Many causes of chronic inflammation were described in CRD and it was revealed that the complications were based on chronic inflammation (16). Increased inflammatory response in CRD is caused by decreased activity of the antioxidant system in addition to oxidative stress (17). Proinflammatory cytokine levels increase due to many cause. These include especially decreased residual levels of renal function and the drugs used in different renal and comorbid diseases (18).

In our study, consistently with the literature, when the patients were evaluated for inflammatory parameters, CRP (**p=0.01**), HsCRP (**p=0.01**), ESR (**p=0.001**), IL-6 (**p=0.001**) and ferritin (**p=0.03**) levels were found to be significantly higher in the patients with advanced stage compared to those with early stage.

Hepcidin is an important mediator associated with both inflammation and iron metabolism, which is synthesized mainly in the liver and also in the heart, skeletal muscle, kidneys and brain (18). The synthesis of the hepcidin, which is a type 2 acute phase protein, is markedly increased in the inflammation and when iron loading is done (19). In the study performed by Tomosugi et al., serum levels of hepcidin were found to be high in the patients with CRD and high levels of hepcidin in these patients were attributed to the presence of chronic inflammation and decreased hepcidin excretion (20).

Again, in different studies, it was found that serum levels of hepcidin were higher and mostly correlated with GFR in the group with CRD (21).

It was reported that, in the patients on hemodialysis, elevated levels of hepcidin were associated with functional iron deficiency, anemia and low grade inflammation (22).

In our study, a significant difference of hepcidin-25 levels was detected between the group with early stage CRD and the group with advanced stage CRD (**p=0.03**). Hepcidin-25 level was significantly higher in the group with advanced stage compared to that with early stage. Consistently with the literature, this may be associated with decreased excretion of hepcidin and increased inflammatory response. Highest levels of hepcidin-25 were observed in the patient group with stage 5 disease.

Hepcidin is a binder for ferroportin and prevents the transportation of the iron. It may be thought that a part of the anemia pathogenesis in CRD may be resulting from the prevention of iron mobilization due to inflammatory iron block caused by elevation of hepcidin levels.

In a study performed by Zaritsky et al., serum levels of ferritin were detected to be the most potent stimulant of the serum hepcidin levels (23).

In our study, in both early stage and advanced CRD groups, statistically significant positive correlation was found between hepcidin-25 levels and ferritin levels (**p< 0.001, p< 0.001**). This was thought to be associated with increase response to inflammation or a mechanism to prevent excessive accumulation of iron that may result from especially increased ferritin concentration.

Although it could not be detected in the patients with early stage, a statistically significant positive correlation was detected between hepcidin-25 level and IL-6 levels in the patients with advanced disease (**p< 0.001**). Upon the stimulation of the macrophage as a result of chronic inflammation, IL-6 is released and then, stimulates mRNA synthesis of the hepcidin in the hepatocytes (16). These findings suggest that, with advanced stage of CRD, the inflammation increases and the correlation between hepcidin-25 and IL-6 becomes more prominent. Nemeth et

al. reported that IL-6 was the key inducer of the hepcidin synthesis in the inflammatory process (8). In the recently published study of Andrews, the investigators demonstrated that IL-6 directly induced hepcidin-25 in the inflammation (24).

In our study, no significant correlation was detected between hepcidin-25 and the inflammatory markers other than IL-6.

In our study, a negative correlation was detected between GFR levels and IL-6 level in the patients with advanced chronic renal disease ($p=0.002$) and this negative correlation was associated with accelerated and exacerbated inflammatory process and increased IL-6 level observed in the advanced stages of the chronic renal disease.

A regression analysis was performed to detect the parameters that mostly affect the anemia in the patient groups and hepcidin and GFR levels were found to be the factors that have the maximum effect on the anemia.

Consequently, in our study, we found that anemia was mostly associated with hepcidin, IL-6 and GFR in the patients with CRD. We believe that functional iron deficiency is related to the correlation across these three parameters. It was evaluated that the increases observed in hepcidin-25 levels and the levels of other inflammatory parameters were associated with inflammatory process occurring in the patients with chronic renal disease. We believe that, especially in the patients who show inadequate response to ESA, there is an inflammatory iron block and the most important marker of this condition, hepcidin-25, may be used as a parameter.

CONCLUSION

There are some findings that supported that hepcidin levels were increased due to decreased GFR resulting from both inflammation and urinary excretion of the hepcidin-25 in the patients with CRD, which may affect the development mechanism of the anemia along with EPO decrease in CRD.

In the patients with anemia associated with chronic renal disease, controlling the inflammation or using the agents that may decrease the levels of hepcidin-25 may be useful in the treatment. More extensive studies are warranted to further support these results.

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