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STUDY ON SERUM HEPCIDIN LEVEL IN CHRONIC KIDNEY DISEASE

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ABSTRACT (Times New Roman, bold, 10)

AIM
The present study was to estimate and to compare serum hepcidin levels in controls and patients of chronic kidney disease and to check the correlation of hepcidin to anemia and inflammation in chronic kidney disease.
OBJECTIVES
Estimate serum hepcidin levels in controls and patients with chronic kidney disease. Determine and correlated to markers of anemia and inflammations in chronic kidney disease.
MATERIALS AND METHODS
Serum Hepcidin was estimated along with other biochemical parameters such as serum Urea, Creatinine, Iron, Ferritin, Transferrin, Total iron binding capacity (TIBC), hsCRP and Hemoglobin.
RESULTS
Hepcidin could be a prognostic marker in the clinical outcome of CKD especially in the progression of CKD.
CONCLUSION
Serum hepcidin correlated positively with markers of iron status (iron and ferritin) in the CKD group

Keywords
Hepcidin, iron, urea ferritin, transferrin, inflammation and erythropoietin.

Introduction

Chronic kidney disease (CKD) refers to an irreversible progressive deterioration in renal function. CKD has become a worldwide, chronic, non-communicable disease epidemic with adverse outcomes of renal failure, cardiovascular disease and premature death. In developed countries, it affects 10 -15% of adult general population. CKD is a chronic inflammatory state which promotes endothelial dysfunction and vascular remodeling. The deteriorating renal function may result in accumulation of uremic toxins which further stimulate inflammation. Anemia is a complication of chronic kidney disease which starts manifesting in the initial stages and increases its prevalence with the progression of CKD. [1-3] The main causes of anemia in CKD are erythropoietin deficiency, iron deficiency and chronic inflammation. The discovery of hepcidin and its functions has led to a better understanding of iron metabolism disorders in CKD. Hepcidin, a small cysteine rich liver-derived peptide. Hormone is the key regulator of systemic iron homeostasis. Altered homeostasis of hepcidin results in various iron disorders. Hepcidin has evolved as the star mediator of anemia of chronic disease and inflammation. Hepcidin functions by causing degradation of the iron transporter ferroportin. Inflammation increases hepcidin production while erythroid activity and hypoxia decrease hepcidin levels. [4-6] Hepcidin levels reflect various key signals involved in iron regulation and it directly controls iron absorption and its bioavailability in circulation. So its measurement should be useful as a clinical tool for the management of iron disorders.

Previous work has shown that serum hepcidin levels were increased in patients with chronic kidney disease who had co-existent anemia. It is possible that changes in hepcidin may underlie the association between anemia and inflammation associated with chronic kidney disease. Increased serum hepcidin levels result in anemia and resistance to erythropoietin stimulating agents. Treatment with anti-hepcidin drugs may improve anemia of CKD. Hence in the present

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study, the serum level of hepcidin was estimated in patients with CKD and the relationship between serum hepcidin, inflammation and anemia were analyzed. [7,8]

MATERIALS AND METHODS

This case control study was conducted in the Department of Biochemistry, Sree Balaji Medical College and Hospital, Chromepet, Chennai during the period of January 2017 – June 2018 among 50 patients of chronic kidney disease visiting the Nephrology outpatient services in the age group of 18 -60 years and 50 healthy age and sex-matched controls. The ground work for the study was started after obtaining clearance from the research committee and the Institutional human ethical committee (Reference number for approval: 002/SBMC /IHEC/2016/835) of Sree Balaji Medical College and Hospital, Chromepet, Chennai.

Age, gender, duration of chronic kidney disease, general history and medications and blood pressure were recorded. Routine clinical examination was done. The study was explained to the participants and informed consent was obtained from them before taking the blood sample. Serum Hepcidin was estimated along with other biochemical parameters such as serum Urea, Creatinine, Iron, Ferritin, Transferrin, Total iron binding capacity (TIBC), hsCRP and Hemoglobin.

SAMPLE SIZE:

Total sample number(n) - 100

GROUP I (50 subjects) - subjects with CKD.

GROUP II (50 subjects) – healthy subjects, selected from people attending Master Health Check-up programme at Sree Balaji Medical College and Hospital.

GROUP I (CASES):

INCLUSION CRITERIA:

Known cases of chronic kidney disease on conservative management in the age group 18 -60 years

EXCLUSION CRITERIA:

Age group less than 18 years and greater than 60 years, Pregnancy, Any malignancy, Pre-existing liver disease and Active inflammatory disease

GROUP II (CONTROLS):

INCLUSION CRITERIA:

Age group of 18-60 years

Clinically healthy individuals

EXCLUSION CRITERIA:

Age group less than 18 years and greater than 60 years, Pre-existing kidney disease, Pre-existing liver disease, Anemia, Active inflammatory state, Pregnancy, any malignancy, Pre-existing cardiac disease and Diabetes mellitus.

INFORMED CONSENT

Patients, who were identified based on inclusion and exclusion criteria as listed above, were invited to participate in the study. They were each provided with an information sheet in either

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English or a vernacular language of their preference. Written consent was obtained from patients who expressed their willingness to participate (enclosed in Annexure II and III). Clinical and socio-demographic data for each patient were obtained in the proforma used (enclosed in Annexure IV).

SAMPLE COLLECTION

After obtaining informed consent, 5 ml of blood was collected from each participant, from a peripheral vein under aseptic precautions in specific vacutainers. Plain tube for hepcidin, urea, creatinine, iron, ferritin, hsCRP and TIBC and EDTA tube for hemoglobin were used. Blood samples collected were used for the estimation of serum levels of hepcidin, urea, creatinine, iron, TIBC and ferritin, hsCRP and hemoglobin. Transferrin saturation value was obtained by calculation.

PROCESSING AND STORAGE OF SAMPLES

Blood collected in the plain tube was allowed to clot, then each tube was centrifuged for 10 minutes at 3500 rpm within 2 hours of blood collection to separate serum. Serum was separated and divided into multiple aliquots. Tubes were labelled with clear sample IDs. Serum sample was used for the estimation of all parameters except hemoglobin for which whole blood was used. Aliquots of serum samples obtained were stored at -23°C. These samples were thawed to room temperature and used for analysis of hs C-reactive protein (hsCRP), iron, hepcidin, ferritin and total iron binding capacity (TIBC) when required.

ESTIMATION OF SERUM HEPCIDIN by ELISA (Enzyme Linked Immuno Sorbent Assay)

Serum hepcidin was measured using a peptide enzyme immune assay kit in Mindray MR-96A fully automated immunoassay analyser.

ESTIMATION OF SERUM UREA by UREASE GLDH- UV METHOD

Estimation of serum urea was done by Urease GLDH- UV method using Mindray BS 390 Fully Automated Analyser.

ESTIMATIONOFSERUMCREATININEBy MODIFIED JAFFE'S METHOD

Estimation of serum creatinine was done in Mindray BS 390 Fully Automated Analyser by Modified Jaffe's Kinetic method.

ESTIMATION OF SERUM FERRITIN by CLIA (Chemi Luminescence Immuno Assay)

Estimation of serum ferritin was done by chemiluminescence assay in Siemens, ADVIA Centaur CP Immunoassay system.

ESTIMATION OF SERUM IRON by COLORIMETRY

Serumironwasmeasuredbycolorimetryin Biosystems semi-automatic analyser BTS-350.

ESTIMATION OF TOTAL IRON BINDING CAPACITY (TIBC) by COLORIMETRY

TIBCwasmeasuredbycolorimetricassayinBiosystems semi-automatic analyser BTS-350.

TRANSFERRIN SATURATION

Transferrin saturation was estimated as the percentage of serum iron to TIBC ratio, as shown

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below:
Transferrin saturation = (Serum iron/ TIBC) *100

ESTIMATION OF SERUM hs C-REACTIVE PROTEIN by CLIA
hs C-reactive protein (CRP) was estimated by done by chemiluminescence assay in Siemens, ADVIA Centaur CP Immunoassay system.

ESTIMATION OF HEMOGLOBIN by COLORIMETRY
Hemoglobin was estimated by colorimetry in automated hematologyanalyser, Mindray BC 5380.

RESULTS
A total of 100 subjects in the age range of 18 to 60 years were selected for the present study. They were divided into two groups. Group I included 50 cases of CKD patients under conservative management and group II included 50 healthy subjects or controls. Levels of serum hepcidin, urea, creatinine, hemoglobin, hs CRP, iron, TIBC, ferritin and transferrin saturation were estimated for all the samples of the study group. The values obtained in cases and controls are presented in the master chart I and II respectively. All the results obtained were statistically analyzed using SPSS software version 16.0. Shapiro-Wilk test was used to test for normality of the data. Mean and standard deviation were used to represent normally distributed data. Median and interquartile ranges were used to represent data which were not normally distributed. Data in the two groups were compared by Kruskal Wallis test. Bivariate correlation analyses were done using Pearson correlation to correlate hepcidin with other parameters. The results of statistical analysis were arranged in tabular form and were plotted in graphs. Kruskal-Wallis Test was used to test the statistical significance of hepcidin and other parameters among the control group and the cases group. The mean values of s . Hepcidin,s. ferritin, s. hsCRP, s. Urea and s. Creatinine levels were higher in group I compared to group II. The mean values of s.Iron, s. TIBC, Transferrin saturation and hemoglobin were lower in group I compared to group I I.

TABLE 1: COMPARISON OF SERUM HEMOGLOBIN LEVEL BETWEEN THE GROUPS

	Group	N	Mean	Std.Deviation	Mean Rank	P value
Hemoglobin (g/dl)	**I**	50	7.324	1.213	25.74	0.000
	II	50	13.922	1.7582	75.26	

The mean value of hemoglobin was significantly lower in CKD group (7.324±1.213 g/dl) compared to control subjects (13.922±1.758g/dl) with $p<0.05$.

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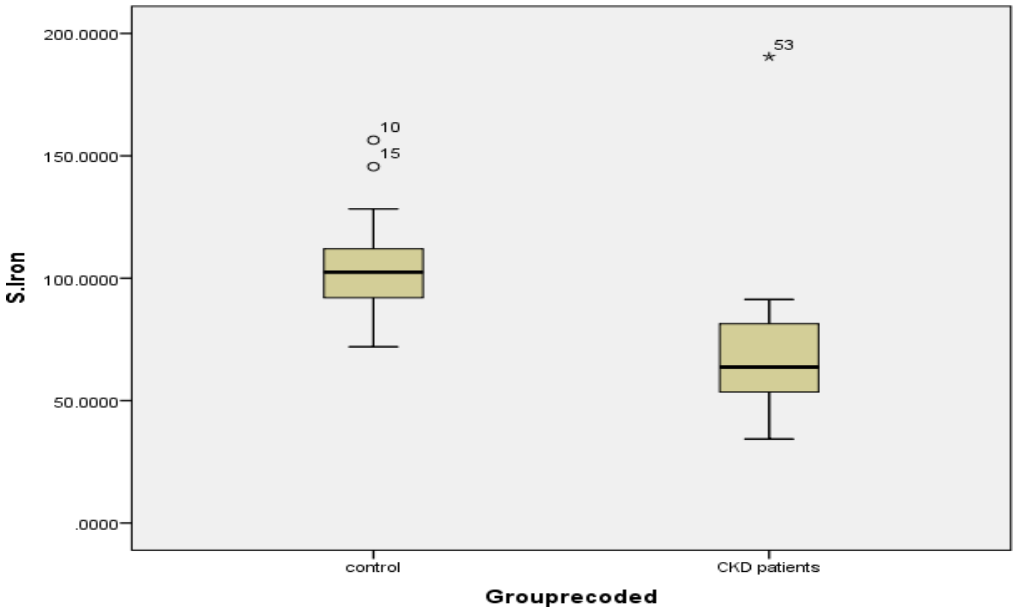
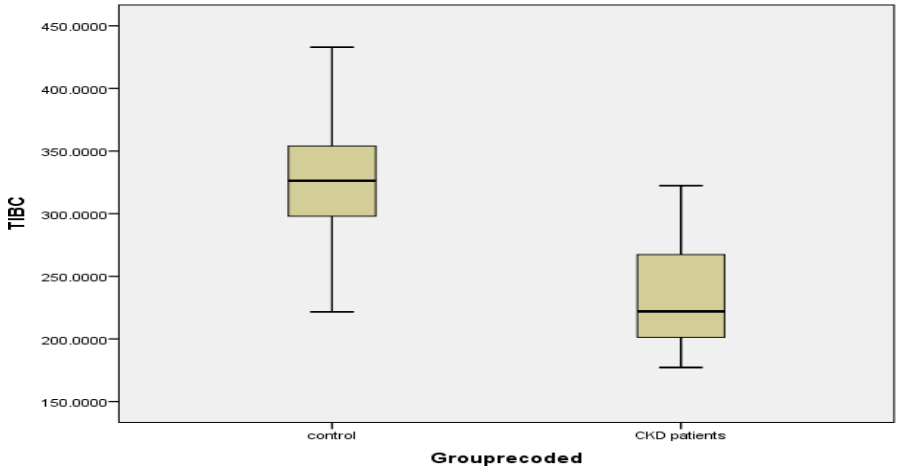
FIGURE 1: SERUMIRONLEVEL INTHESTUDY GROUP



Data are shown in the form of box and whisker plots, with quartiles and medians shown. An outlier is shown as a numbered dot.
The mean value of Iron stores was significantly lower in CKD (67.371±23.909 µg/dl) compared to control subjects (103.166±17.293 µg/dl) with p <0.05.

FIGURE 2: SERUMTIBC LEVEL IN THE STUDY GROUP



Data are shown in the form of box and whisker plots, with quartiles and medians shown. The mean value of s. TIBC was significantly lower in CKD group (236.964± 41.066 µg/dl) compared to control subjects (328.812 ± 45.013 µg/dl) with p <0.05.

FIGURE 3: SERUM TRANSFERRIN SATURATION IN THE STUDY GROUP

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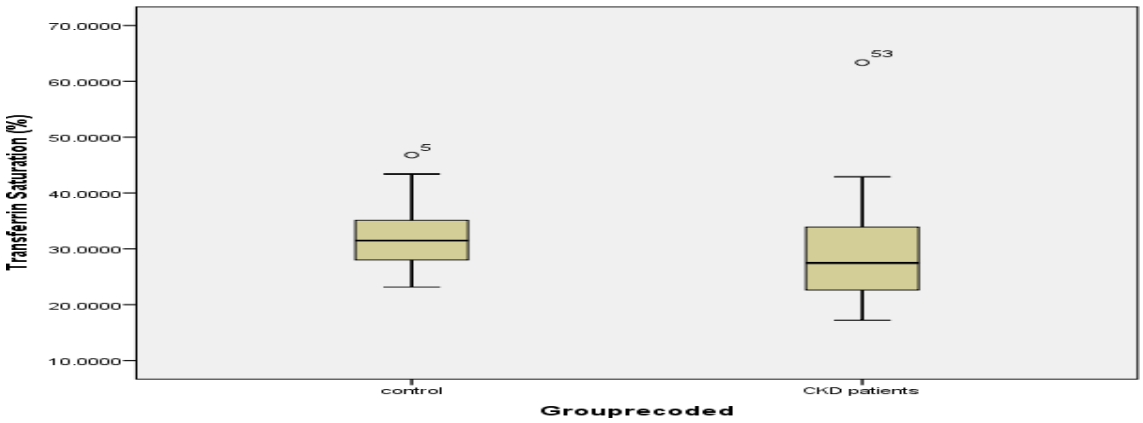


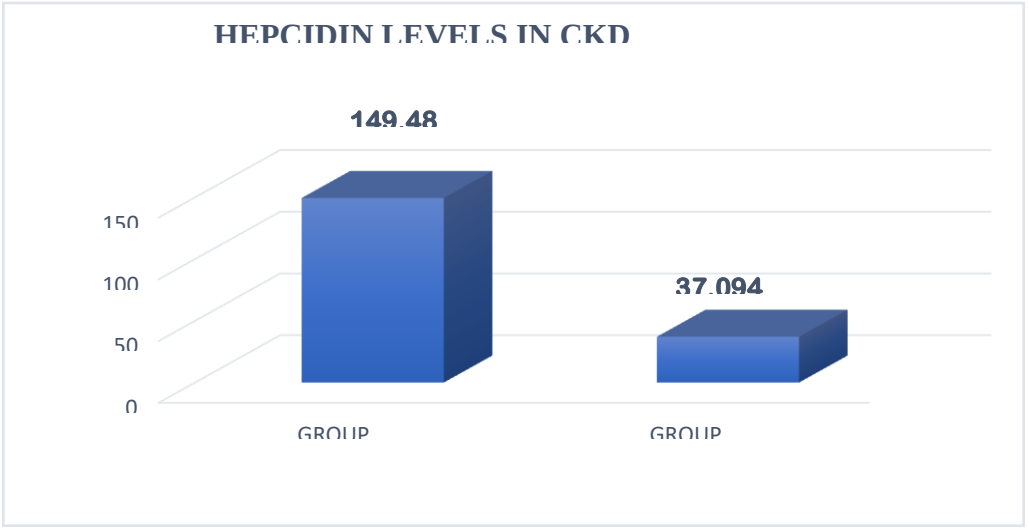
Data are shown in the form of box and whisker plots, with quartiles and medians shown. An outlier is shown as a numbered dot.

The mean value of transferrin saturation was lower in CKD group (28.454 ± 8.048 %) compared to control subjects (31.65 ± 5.294 %) and it was statistically insignificant between the two groups with p =0.07 (p>0.05).

TABLE2:COMPARISONOFSERUMFERRITIN LEVEL BETWEEN THE GROUPS

	Group	N	Mean	Std. Deviation	Mean Rank	P value
S.Ferritin (ng/ml)	**I**	50	187.757	93.1905	75.22	0.000
	II	50	69.762	24.1829	25.78	

FIGURE 4: GRAPHICAL REPRESENTATION OF HEPCIDIN LEVELS IN THE TWO GROUPS



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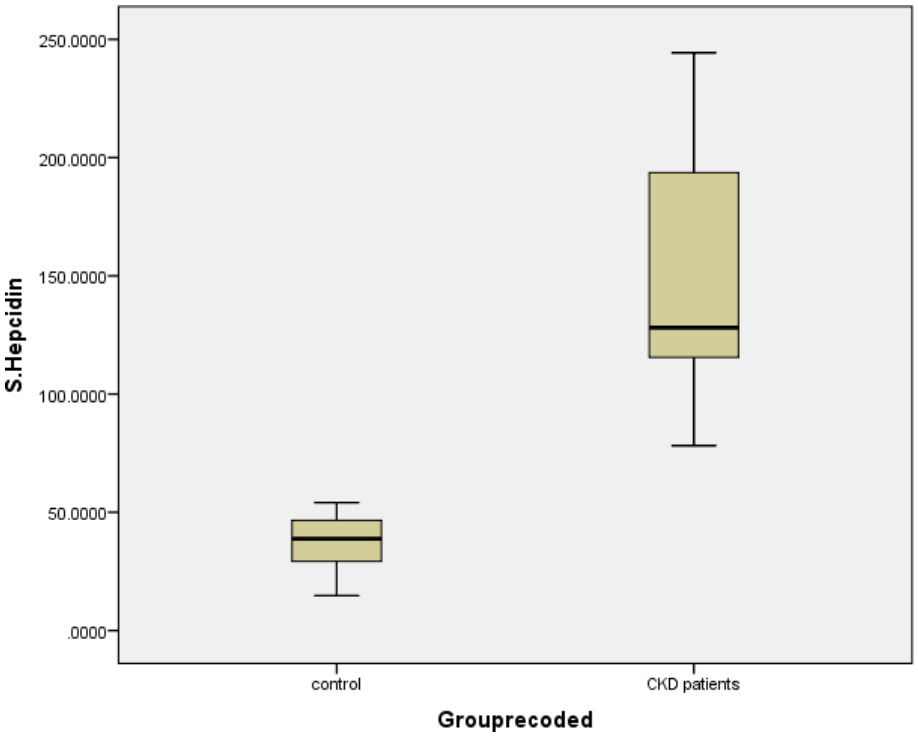
The mean value of s. Hepcidin level was significantly higher in Group II- CKD group (149.484 ± 47.539 ng/ml) compared to Group I control group (37.094 ± 12.073 ng/ml).

FIGURE 5: SERUM HEPCIDIN LEVEL IN THE STUDY GROUP



Data are shown in the form of box and whisker plots, with quartiles and medians shown.
Figures 8 and 9 show that the mean value. Hepcidin was significantly higher in CKD patients (149.484± 47.539 ng/ml) compared to control subjects (37.094± 12.073 ng/ml) with p <0.05.

CORRELATION ANALYSIS

Correlation analysis was done on the data obtained. The data are represented as scatterplots with correlation coefficient (r) and p values shown.

TABLE 3: PEARSON'S CORRELATION BETWEEN HEPCIDIN AND OTHER PARAMETERS AMONG CKD CASES (GROUP II)

VARIABLES		S. HEPCIDIN
S. FERRITIN	r value	0.907
	p value	0.000
S. hsCRP	r value	0.942
	p value	0.000
S. IRON	r value	-.490
	p value	0.000

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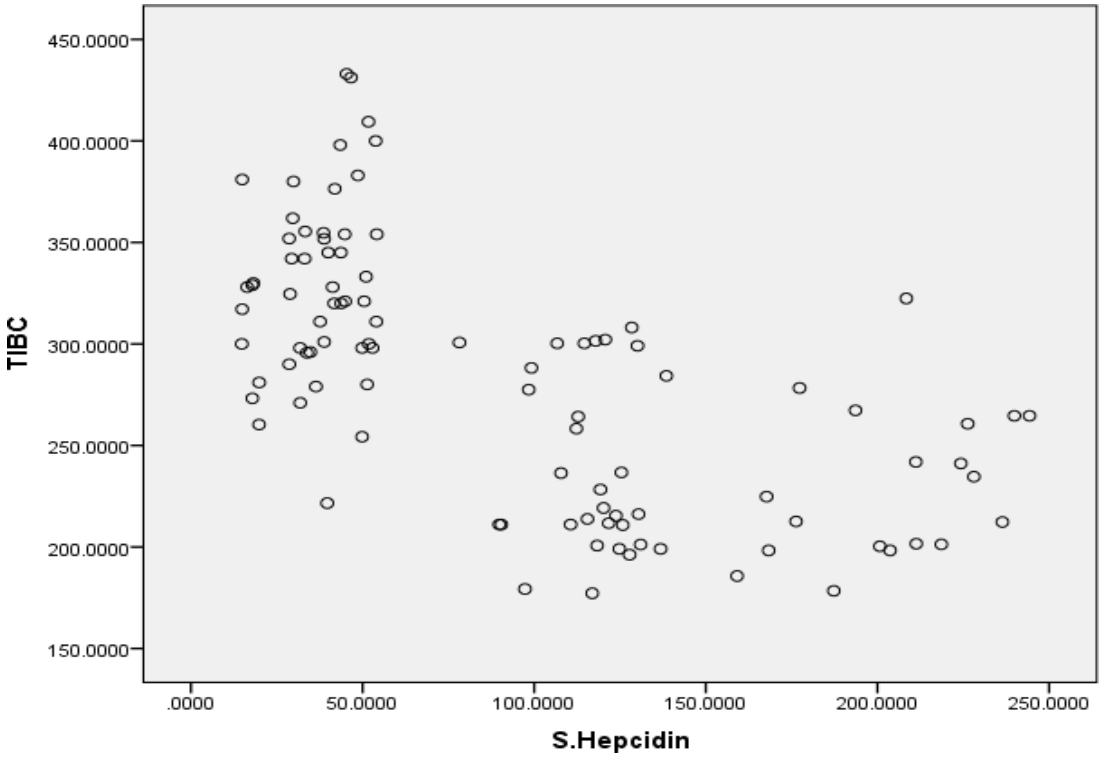
S. TIBC	**r value**	-.627
	p value	0.000
S. TRANSFERRIN	**r value**	-0.491
	p value	0.070
S. UREA	**r value**	0.403
	p value	0.000
S. CREATININE	**r value**	0.843
	p value	0.000
S.HEMOGLOBIN	**r value**	-0.724
	p value	0.000

**. Correlation is significant at the 0.01 level (2 -tailed).

The mean value of s. hepcidin was found to have strong positive correlation with the mean values of s. ferritin, s. hsCRP and s. creatinine and strong negative correlation with hemoglobin with r-value > 0.7.

FIGURE 6: SCATTER PLOT OF SERUM HEPCIDIN VS TIBC



Thereisamoderatenegativecorrelationbetween serum hepcidin and serum TIBC with r- value = -0.627.

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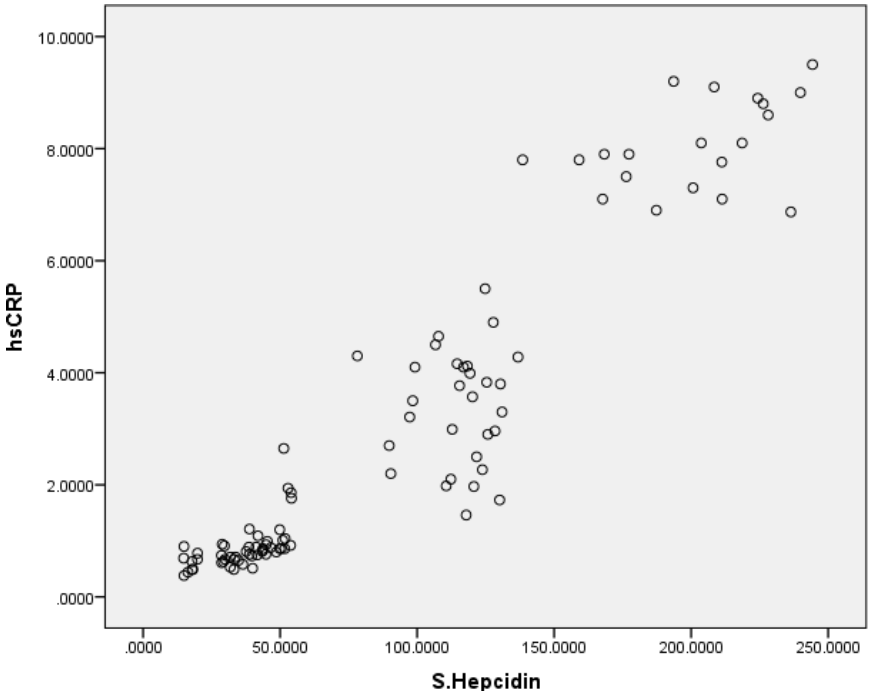
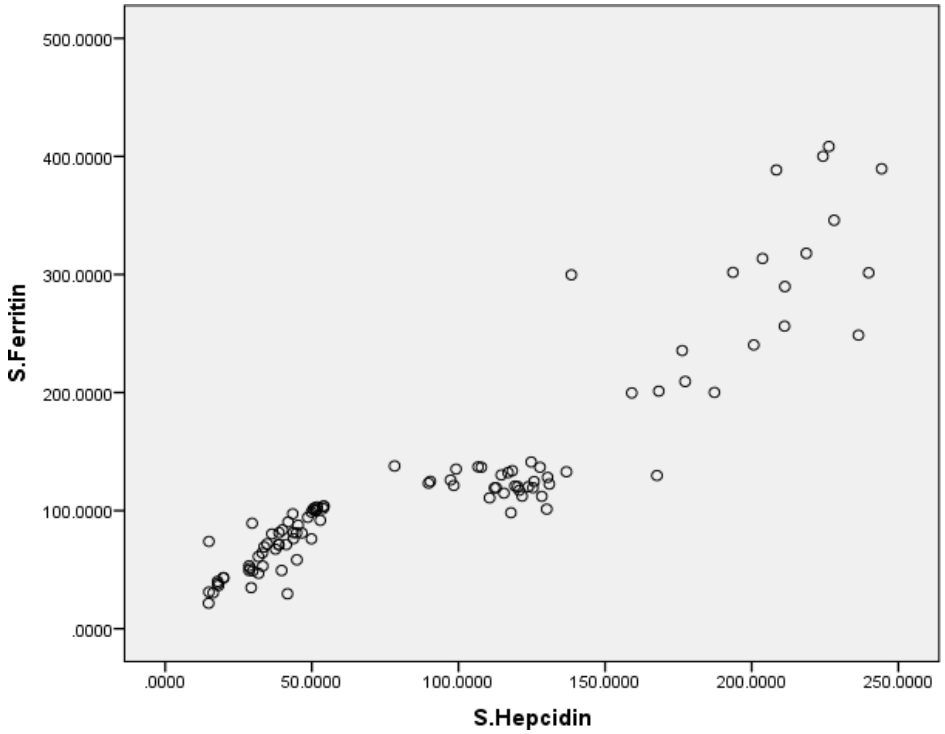
FIGURE 7: SCATTER PLOT OF SERUM HEPCIDIN VS FERRITIN



Serum hepcidin and serum ferritin values were found to have a significant positive correlation with r- value = 0.907.

FIGURE 8: SCATTER PLOT OF SERUM HEPCIDIN VS hsCRP



Thereisastrongpositivecorrelationbetween hepcidin and hsCRP with r- value = 0.942.

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DISCUSSION

In the present study, a total of 100 subjects were studied, of these, 50 were diagnosed to have chronic kidney disease and 50 healthy subjects who served as controls. Hepcidin levels were significantly higher in patients with CKD than in control subjects. Serum urea, creatinine, ferritin and hs CRP were found to be elevated in CKD cases.[9,10] Serum iron, TIBC, transferrin saturation and hemoglobin were decreased in the control group. S. urea and S. creatinine were found to be elevated in the CKD group compared to the control group. The mean value of S. urea and S. creatinine were 24.08 ± 7.64 mg/dl and 0 .762 ± 0.219 mg/dl in the control group respectively while in CKD group, the mean value of S. urea and S. creatinine were 93.98± 32.876 mg/dl and 8.578 ± 3.88 mg/dl respectively. Both parameters were found to have significant positive correlation with hepcidin with $p<0.001$. These parameters are known to increase with the progression of CKD.[11] In the present study, serum hepcidin concentrations were found to be significantly increased in patients with CKD [Mean value: 149.484 ± 47.539 ng/mL] when compared to the control group [Mean value: 37.094± 12.073 ng/mL; $p<0.001$]. Serum hepcidin levels were found to be increasing with the progression of CKD. This finding is supported further by the highly significant positive correlation observed between serum hepcidin and creatinine in CKD cases ($r= 0.8437$, $p<0.001$). These findings are in accordance with the study of Tarek et al, which reported an increase in serum hepcidin levels in all stages of CKD among 54 CKD patients under conservative management and 40 CKD patients under hemodialysis.[12-15]

Hepcidin is cleared from the body by the kidneys. The increase in hepcidin seen in CKD is due to its reduced renal clearance associated with the deteriorating renal function. Another reason could be the chronic inflammatory state of CKD which stimulates hepcidin production.[16] Uremia is a state of heightened inflammatory activation which results in IL-6 stimulation and finally in increased hepcidin production. Increased hepcidin levels cause iron-restricted erythropoiesis and final results in anemia in CKD. S. Iron, TIBC and transferrin saturation were found to be decreased in CKD group compared to controls. Among the control group, the mean value of S. Iron was observed to be 103.166 ± 17.293 µg/dl and in CKD patients, it was 67.371 ± 23.909 µg/dl. Hepcidin and iron had a moderate negative correlation with r- value being-0.490 and p value.

In this study, hepcidin and ferritin were significantly higher in chronic kidney disease patients compared to control subjects. The mean value of ferritin was 69.762 ± 24.182 ng/ml in control group whereas for the CKD group it was 187.757 ± 93.19 ng/ml. Also, Malyszko et al found that in his study on patients with chronic renal failure and hemodialyzed patients that serum ferritin and hepcidin were significantly higher than in the healthy volunteers.[17] There was a strong positive correlation between ferritin and hepcidin among the CKD cases($r = 0.907$, $p<0.001$) which is in accordance with the study of Mercadal et al. He observed a positive correlation between hepcidin and ferritin in his study among 199 non-dialysed non- transplanted patients with CKD stages 1 -5. Hepcidin level of the two study groups in the present study was related to ferritin that agrees with the results of Peters et al, who revealed that serum ferritin concentration was a significant predictor of hepcidin-25 levels in CKD by means of multiple regression analysis. Similar correlation between ferritin and hepcidin was reported by Dallalio et al, in anemic patients undergoing diagnostic bone marrow examination. However, Ma lyszko et al could not find a significant correlation between hepcidin and ferritin in his study population of patients with chronic renal failure on conservative treatment and on hemodialysis.[17,18]

CKD is a state of chronic persistent low-grade inflammation with persistent elevation of pro-inflammatory markers. The prototype marker of inflammation in the clinical setting is hsCRP, a

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positive acute phase reactant and higher level of this inflammatory biomarker is associated with cardiovascular mortality in patients with renal insufficiency.[19] The elevated levels of serum hepcidin in patients with CKD indicate an underlying inflammatory state associated with advanced renal failure, with loss of renal function, and development of anemia of chronic disease, the thing that was obvious in the present study in the form of high hsCRP in chronic kidney disease patients when compared to control [Mean level: Cases - 5.25 ± 2.50 mg/L; Controls - 0.875 ± 0.407 mg/ L; $p<0.001$]. This is similar to the finding of Carmen et al. He observed a positive correlation between hsCRP and hepcidin in anemic CKD patients. However, he could not establish any relation between hepcidin and hsCRP among non-anemic CKD patients. As the renal function declined, there was a progressive increase in the hsCRP levels. Further a strong positive correlation was found between hepcidin and hsCRP levels ($r=0.942$; $p<0.001$) which shows that hepcidin is a positive acute phase reactant. Similar finding of positive correlation between hsCRP and hepcidin was observed by Karthik al and Tarek et al in their studies as well. [20,21]

Anemia among the present study population could be either due to iron deficiency or anemia of chronic disease. With the data available, it is difficult to differentiate between iron deficiency anemia and anemia of chronic disease. Measuring soluble transferrin receptor-log ferritin ratio (sTfR/log ferritin ratio) would be useful to differentiate between the two conditions. Even though soluble transferrin receptor expression is shown to be negatively affected by inflammation, the sTfR/log ferritin ratio is considered as an accurate indicator of body iron stores in the presence of inflammation.[22]

Taken together, the results of the present study suggest that hepcidin levels are elevated in CKD and hepcidin is a predictor of iron stores and inflammation since it correlated well with the inflammatory marker hsCRP and markers of iron status, serum iron and ferritin levels. This increase in hepcidin levels reflects both the renal impairment leading to reduced renal clearance of hepcidin and the state of chronic inflammation. These findings highlight the close relationship between inflammation and anemia in CKD. [23] 100 participants who were divided into two groups were involved in this study. The outcomes of serum hepcidin, s. urea, s. creatinine, s. iron stores, s. TIBC, Transferrin saturation, s. ferritin, hemoglobin and s. hsCRP were compared between the two groups. All these parameters significantly correlated among the groups with the level of significance $p<0.05\%$ except transferrin saturation. Patients of CKD had higher serum levels of hepcidin, hscrp and ferritin and lower iron, TIBC and transferrin saturation compared to control. There was strong positive correlation between hepcidin with ferritin and hepcidin and hsCRP. Thus, hepcidin could be considered as the link between inflammation and anemia associated with CKD.

CONCLUSION

In this study comprising Serum hepcidin levels were significantly increased in patients with CKD than in control subjects. Serum hepcidin correlated positively with markers of iron status (iron and ferritin) in the CKD group. Serum hepcidin correlated positively withthe inflammatory marker hsCRP in the CKD group. Our present study indicated that serum hepcidin provides useful information about the level and availability of iron during inflammation as compared with traditional markers of iron status. Hence, it appears that hepcidin could be a prognostic marker in the clinical outcome of CKD especially in the progression of CKD.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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