STUDY OF THE CORRELATION OF HEPCIDIN IN ABSOLUTE AND FUNCTIONAL IRON DEFICIENCY ANEMIA IN CKD PATIENTS

THESIS

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In partial fulfilment of the requirement for the degree of

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(NEPHROLOGY)

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DECLARATION

I hereby declare that the thesis titled "**Study of the correlation of hepcidin in absolute and functional iron deficiency anemia in CKD patients**" embodies the original work carried out by the undersigned in All India Institute of Medical Sciences, Jodhpur.

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CERTIFICATE

This is to certify that the thesis titled "**Study of the correlation of hepcidin in absolute and functional iron deficiency anemia in CKD patients**" is the bonafide work of **Dr. Santosh Kumar V** carried out under our guidance and supervision, in the Department of Nephrology, All India Institute of Medical Sciences, Jodhpur.

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Dr. Santosh Kumar V

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LIST OF ABBREVIATION

CKD	CHRONIC KIDNEY DISEASE
ESRD	END STAGE RENAL DISEASE
IDA	IRON DEFICIENCY ANAEMIA
KDOQI	KIDNEY DISEASE OUTCOMES QUALITY INITIATIVE
KDIGO	KIDNEY DISEASE: IMPROVING GLOBAL OUTCOMES
HB	HEMOGLOBIN
HD	HEMODIALYSYIS
PD	PERITONEAL DIALYSIS
GFR	GLOMERULAR FILTERATION RATE
AST	ASPARTATE TRANSAMINASE
ALT	ALANINE TRANSAMINASE
RETI HE	RETICULOCYTE HEMOGLOBIN EQUIVALENT
CAKUT	CONGENITAL ANOMALY OF KIDNEY AND URINARY TRACT
TSAT	TRANSFERRIN SATURATION
TIBC	TOTAL IRON BINDING CAPACITY
ELISA	ENZYME LINKED IMMUNOSORBENT ASSAY
WCX-TOF-MS	WEAK CATION EXCHANGE TIME-OF-FLIGHT MASS SPECTROMETRY
EPO	ERYTHROPOIETIN
FIDA	FUNCTIONAL IRON DEFICIENCY ANAMEIA

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SUMMARY

Background: In chronic kidney disease, inflammation and impaired renal clearance increase plasma hepcidin, inhibiting duodenal iron absorption and sequestering iron in macrophages. These effects of hepcidin can cause systemic iron deficiency, decreased availability of iron for erythropoiesis, and resistance to endogenous and exogenous erythropoietin. Together with impaired renal production of erythropoietin, hepcidin-mediated iron restriction contributes to anemia of chronic kidney disease. In patients with systemic inflammation and high hepcidin concentrations, even when iron stores are adequate and sufficient for baseline erythropoiesis, iron cannot be released from stores rapidly enough to meet the needs of pharmacologically stimulated erythropoiesis. This condition is referred to as functional iron deficiency.

Aims and objectives: The study is aimed to correlate Serum Hepcidin levels with Absolute and functional IDA parameters in chronic kidney disease patients.

Methods: This was as Observational and Cross-Sectional Study conducted in our department involving CKD patients with anemia who underwent evaluation for anemia and were diagnosed to have absolute and functional iron deficiency based on Iron, TSAT, TIBC, ferritin and Ret-He levels. Serum Hepcidin was estimated using ELISA method. Hepcidin level was compared across different stage of CKD along with other parameters of iron deficiency anemia. Analysis was also done to compare Hepcidin levels with Ret-He levels across different stages of CKD to establish relationship between Hepcidin, Ret-He and Functional iron deficiency anemia. Comparison of various parameters which contribute towards anemia in advanced CKD in the form of Calcium, Phosphorous, Intact PTH, ESR, CRP and ferritin was also done with Hepcidin to establish correlation in different stages of CKD.

Results: In this study, 139 patients were enrolled out of which 88 (70.5%) were males while the rest 51(36.65%) were females. Majority of patients were in CKD stage V (60) where the mean age was 41 years, stage IIIA, IIIB, IV had 13,18 and 41 patients respectively. Mean hepcidin levels in CKD stage II was 190.57, in stage IIIA was 199.23, stage IIIB was 275.56, stage IV was 409.56 and in stage V was 543.47. The increase in mean hepcidin levels in advancing stages of CKD was statistically significant ($p < 0.05$). Mean RET HE levels in CKD stage II was 27.43, in stage IIIa

was 27.38, stage IIIB was 24, stage IV was 20.02 and in stage V was 16.35. The decrease in mean RET HE levels in advancing stages of CKD was statistically significant ($p < 0.05$). The mean Hemoglobin of study participants in participants with absolute iron deficiency was 9.74 ± 1.37 and in participants with functional iron deficiency was 7.36 ± 1.62. The mean hepcidin levels of study participants in participants with absolute iron deficiency was 259.19 ± 108.68 and in participants with functional iron deficiency was 517.99 ± 117.48.(p value-<0.05). The mean RET HE of study participants in participants with absolute iron deficiency was 24.45 ± 6.79 and in participants with functional iron deficiency was 17.28 ± 5.33.

Conclusions: Hepcidin is a key regulator of iron homeostasis and plays a role in the pathogenesis of anemia of chronic disease. Its levels are increased in patients with chronic kidney disease (CKD) due to diminished renal clearance and an inflammatory state. Increased hepcidin levels in CKD patients are supposed to be responsible for functional iron deficiency in these patients and contribute to renal anemia and resistance to erythropoiesis-stimulating agents. Given the role of hepcidin in the pathophysiology of functional iron deficiency of anemia in CKD patients, in future, it can be considered as a practical diagnostic tool guiding management of renal anemia, and its possible usefulness as a prognostic biomarker.

INTRODUCTION

Chronic kidney disease (CKD) is a worldwide public health problem, with adverse outcomes of kidney failure, cardiovascular disease (CVD), and premature death.

Definition of Chronic Kidney Disease Criteria

1. Kidney damage for 3 months, as defined by structural or functional abnormalities of the kidney, with or without decreased GFR that can lead to decreased GFR, manifest by either:

- Pathological abnormalities; or
- Markers of kidney damage, including abnormalities in the composition of the Blood or urine, or abnormalities in imaging tests

2. GFR <60 mL /min/1.73 m2 for ≥3 months, with or without kidney damage [1].

Stages of Chronic Kidney Disease [1].

Stage	Description	GFR (ml/min/1.73m²)
1	Kidney damage with normal or ↑ GFR	≥ 90
2	Kidney damage with mild ↓ GFR	60-89
3	Moderate ↓ GFR	30-59
4	Severe ↓ GFR	15-29
5	Kidney failure	<15

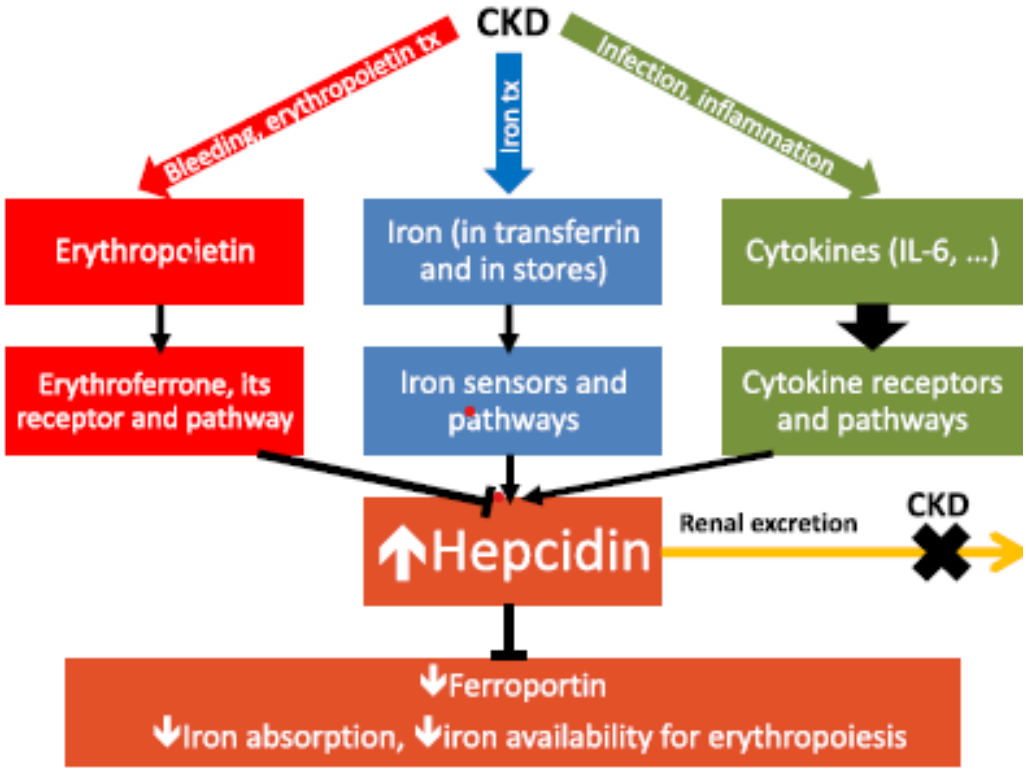
Anemia is an anticipated consequence as renal function declines, and generally begins to develop before ESRD. The severity of anemia however increases with declining kidney function. [2]

The two main factors that contribute to the development of anemia in CKD include erythropoietin (EPO) deficiency [3] and dysfunctional iron metabolism [4]. Until now, there have been several attempts to normalize hemoglobin levels in CKD by replacing EPO, which have ultimately failed [5, 6]. Subsequent analysis has suggested that EPO resistance is the key cause of the disappointing results [7]. Among others, functional or true iron deficiency is the most important cause of EPO resistance [8], and hepcidin is thought to be the fundamental peptide of EPO resistance by sequestrating iron into storage sites in CKD patients Hepcidin is being extensively studied for its

association with anemia in CKD where it has also been associated with inflammation. Hepcidin is thought to be the major regulator of dietary iron absorption and cellular iron release from macrophages, and it exerts its regulatory function by counteracting the function of ferroportin, the major cellular iron exporter. Hepcidin induces internalization and degradation of ferroportin, which results in increased intracellular iron stores, decreased dietary iron absorption, and decreased circulating iron levels, which may be the cause for functional iron deficiency (FID) [9]

Hepcidin lowers the available serum iron levels by limiting iron efflux from the body's iron stores; [10] therefore; it is plausible that iron might be sequestrated in iron stores as the serum hepcidin level increases. This may cause bone marrow iron deficiency despite sufficient iron in storage sites, [11] suggesting that sufficient serum levels of TSAT and ferritin may not guarantee sufficient production of RBC when the serum hepcidin level is increased. Four mechanisms play a role in determining the value of hepcidin, i.e., regulation by iron status, hypoxia, inflammation, and erythropoietic signals

The peptide is produced in the liver and acts as a normal regulator of hemostasis and a negative regulator of iron absorption in normal conditions. High levels of hepcidin inhibit iron absorption from duodenum and iron release from macrophages (12). It is generally accepted that oral iron intake can increase the levels of hepcidin in normal individuals. Whereas iron deficiency can severely affect the levels of hepcidin in mice (13). Acute type II inflammatory reactants, associated with interleukin 6, are shown to be responsible for hepcidin production. In anemia, erythropoietin and hypoxia resulted in increasing of iron absorption while reducing hepcidin production (14). The occurrence of anemia in normal individuals decreases hepcidin levels. Therefore, regarding the level of anemia, normal levels of hepcidin are considered high in patients with CKD. Moreover, in healthy erythropoietin-receiving individuals, the levels of hepcidin decrease to 70%-75% in 24 hours.



Picture 1: Role of Hepcidin in Iron Metabolism

In addition to its anti-microbial properties, it has also been found to be a key regulator of iron utilization, providing increased understanding of why chronic kidney disease patients absorb iron poorly from the gut and why many hemodialysis patients develop functional iron deficiency in the presence of inflammation. Hepcidin synthesis is upregulated in uremia, as in other inflammatory states. The ability to measure hepcidin in biologic fluids has stimulated interest in the potential applicability of this measurement as a more informative marker of iron status than the traditional iron indices such as serum ferritin and transferrin saturation. Until recently, however, the assays for measuring hepcidin have lacked precision, accuracy, and internal validation. Over the last few years, however, several assays have become available that address these limitations. Broadly speaking, these can be divided into radioimmunoassay, ELISAs, and mass spectrometry methods.

The increased hepcidin levels seen across the spectrum of CKD have important implications for anemia management in CKD. As renal function worsens, there is an increased need for EPO administration along with either supplemental oral or parenteral

iron. In CKD, increased inflammation and possibly decreased clearance of hepcidin can lead to higher serum hepcidin levels, further contributing to iron-restricted erythropoiesis and EPO resistance. Thus, high hepcidin levels could predict the need for parenteral iron to help overcome hepcidin-mediated iron-restricted erythropoiesis and the need for relatively higher EPO doses to suppress hepcidin production. Conversely, patients with low hepcidin would be expected to respond better to oral iron. If so, hepcidin concentrations may become a unique biomarker to guide iron therapy in CKD.

ABSOLUTE VERSUS FUNCTIONAL IRON DEFICIENCY

It is important to differentiate between absolute (or storage) iron deficiency and functional (or relative) iron deficiency. In absolute iron deficiency, the total body iron stores are depleted, limiting the production of RBCs. Contributing factors to absolute iron deficiency include decreased gastrointestinal absorption in patients with CKD and increased blood loss (for example in the setting of uremia-induced platelet dysfunction and the iatrogenic loss from serial blood draws or access-site and circuit issues during the dialysis procedure).[15]

By contrast, functional iron deficiency occurs due to inefficient utilization of iron stores, stemming from one or both of two main phenomena. The first of these, anemia of chronic inflammation, is known as reticuloendothelial cell iron blockade. This may occur in the absence of EPO supplementation and can occur in inflammatory diseases other than CKD. Specifically, reticuloendothelial cell iron blockade can be triggered by active infection or inflammation, hypoxia, or genetic deficiencies.[16] The second process relates to the use of exogenous EPO. Because RBC production increases in response to ESAs, the available iron may be used faster than the existing iron stores are able to release it, leading to a supply/ demand mismatch and a “relative” iron deficiency.[17,18]

Conventional methods for diagnosing iron deficiency involve measurement of serum iron, ferritin levels, and TSAT, but both are indirect markers. Among these, serum ferritin, which reflects the total amount of body iron stores, is a universally available and standardized measurement and is the most effective test for detecting iron

deficiency. However, false-positive serum ferritin values can be observed because they are influenced by inflammation or inflammation, malignancy, or liver disease. Serum iron and TIBC were used to calculate the percentage transferrin saturation (TSAT). Values below 20%, and certainly below 18%, indicate an inadequate supply of iron for hemoglobin synthesis and red blood cell production. Plasma transferrin is a glycoprotein with two iron-binding domains and is synthesized by the liver. It is the most important vehicle for transporting iron into cells and preventing iron-mediated free radical toxicity. In the treatment of CKD patients, low TSAT (<20%) combined with low serum ferritin is diagnostic of absolute iron deficiency. A low TSAT combined with normal or high serum ferritin is diagnostic of functional iron deficiency.[19] In general, serum ferritin <100 ng/mL and/or TSAT <20% is defined as absolute iron deficiency and serum ferritin 100 ng/mL and/or TSAT <20% is considered functional iron deficiency. In CKD patients, serum ferritin levels for absolute iron deficiency are higher because of chronic inflammation, infection, malnutrition, or malignancy and not always due to iron overload [20]

Reticulocyte Hb equivalent (RET- He) levels <25 picograms indicate classic iron deficiency and predict functional iron deficiency in those receiving Erythropoiesis Stimulating Agent (ESA) therapy. RET-He value < 30.6 picograms seems to be the best predictive value for the possible response to intravenous iron therapy in chronic kidney disease (CKD) patients undergoing hemodialysis. RET-He, a new hematological parameter in routine blood tests, and may play an important role in the differential diagnosis of hypochromic microcytic anemia[18] RET-He measures hemoglobin in reticulocytes that are released from the bone marrow approximately 18 to 36 hours before they form into mature erythrocytes. Therefore, the RET-He parameter offers a direct index of iron availability. Thus, RET-He has considerable ability to provide high accuracy readings of the iron status of HD patients [21]

REVIEW OF LITERATURE

During the search of literature pertaining to correlates of cardiovascular morbidity in IDA in ESRD patients, there is dearth of data from Indian population and majority of data is from western countries.

A study by ***Łukaszyk E et al*** showed that Absolute iron deficiency was present in 17% of the patients, functional iron deficiency was present in 12% of the patients. Functional iron deficiency was associated with significantly higher serum levels of fibrinogen, ferritin, transferrin saturation, total iron binding capacity, hepcidin and older age relative to patients with absolute iron deficiency. [22]

A study by ***Norishi Ueda et al*** showed that Serum inflammation markers (CRP and IL-6), ferritin and hepcidin were increased in HD patients with FIDA .Iron absorption was reduced in HD patients with FIDA.FIDA in HD adults could be managed by Intravenous iron therapy but not oral iron therapy, the overall response rate to IV iron therapy was only 46.3% in HD adults with FIDA, while IV iron therapy produced a significant but only small increase in Hb (mean, 0.54 g/dL). , IV iron Therapy increased serum hepcidin levels, which in turn exacerbated FIDA, requiring higher doses of IV iron to maintain Hb , these observations indicate that as CKD advances, inflammation worsens and increases ferritin and hepcidin, leading to inhibition of iron absorption and efflux and subsequent hypo responsiveness to iron therapy. As a result, higher dose of IV iron may be required for the management of IDA in CKD patients with apparent inflammation [23]

In a study by ***Nalado, AM et al***, an interplay between emerging biomarkers of inflammation and anemia has been shown to play a vital role in the increased risk of cardiovascular disease in CKD patients. This association may partly be responsible for the persistent increased risk of mortality despite correction of anemia in CKD patients. Patients with high levels of hepcidin were up to 6 times more at risk of dying compared to patients having normal levels of hepcidin (HR: 6.14; $P<0.001$). Levels of GDF-15 and iron deficiency anemia were not associated with mortality in the CKD patients. Plasma higher levels of hepcidin were strongly associated with CKD progression when compared to normal hepcidin levels [24]

Sonkar SK et al found significantly high level of hepcidin (P <0.05) and high-sensitivity C-reactive protein (CRP) (P <0.05) in Functional iron deficiency as compared to Absolute iron deficiency as well as normal iron level. They also found other inflammatory markers such as albumin, transferrin, and ferritin to be significantly associated with FID [25]

Goyal H et al observed that while absolute iron deficiency (transferrin saturation <20%, ferritin <40 ng/ml) is associated with downregulation of hepcidin. Almost similar association persisted when cut-off value for serum ferritin was raised to 100 ng/ml as per NKF/KDOQI 2006 clinical practice guidelines except that no association was observed in absolute iron deficiency category. Cut-off value for hepcidin for differentiating absolute iron deficiency from other categories in our study population is ≤ 34 ng/ml (area under curve = 0.836, P< 0.0001). Serum hepcidin level is increased in non dialysis CKD patients as compared to healthy adults possibly due to associated inflammation and decreased renal clearance. Furthermore, iron status modifies hepcidin level and its association with Hb. Raised hepcidin can predict the need for parenteral iron therapy and need for higher dose of recombinant human EPO to overcome iron-restricted erythropoiesis.[26]

The human hepcidin gene (*HANP*) encodes a precursor protein of 84 amino acids, preprohepcidin. Preprohepcidin undergoes enzymatic cleavage, resulting in a protein of 64 amino acids, prohepcidin. The biologically active 25-amino acid form, hepcidin-25, is then produced by post-translational processing. Additional degradation results in the production of two isoforms, hepcidin-20, and hepcidin-22. Measurement of serum hepcidin levels was a major issue until recently. In animal studies, hepcidin mRNA expression in the liver has been the preferred method. Many human studies have already been published using a commercially available enzyme- linked immunosorbent assay kit that uses antibodies directed against prohepcidin. However, the diagnostic use and interpretation of the data obtained by these measurements have been controversial because serum prohepcidin levels may not correlate well with those of hepcidin-25, the active form. Measurement of hepcidin-25 needed to overcome several technical di6culties, such as small size and limited availability of the antigen.

In a study by ***Ashby et al.***, hepcidin- 25 level was inversely correlated with estimated glomerular filtration rate in CKD patients and was reduced by erythropoietin

therapy, consistent with previous reports. Nevertheless, contrary to the hypothesis that hepcidin may be a useful predictor of erythropoietin resistance, there was no correlation between hepcidin-25 levels and erythropoietin dose.[27]

The authors also demonstrated conflicting data with their new specific assay for hepcidin-25:

(1) Healthy subjects had a diurnal pattern of hepcidin, but patients with CKD, regardless of dialysis, showed no clear diurnal pattern.

(2) The relationship between hepcidin and ferritin was confirmed in predialysis patients, but the relationship was no longer observed in hemodialysis patients.

(3) Although inflammation has been well recognized to increase hepcidin production, there was no correlation between hepcidin and inflammation markers such as CRP and interleukin-6 in predialysis patients

In multivariate analysis by ***Mercadel et al***, absolute iron deficiency was associated with lower hepcidin values, and inflammation combined with a normal or functional iron profile with higher values, independent of other determinants of hepcidin concentration, including EPO, GFR, and albuminuria. The hepcidin level, although it rose overall when GFR declined, collapsed in patients with absolute iron deficiency. There was a significant interaction with iron status in the association between Hb and hepcidin. Except in absolute iron deficiency, hepcidin's negative association with Hb level indicates that it is not downregulated in CKD anemia. [28]

Aya Eguchi et al investigated the iron status and serum hepcidin levels of peritoneal dialysis (PD) patients who had not received frequent doses of an erythrocytosis-stimulating agent (ESA) and had not received iron therapy. Their serum hepcidin levels were significantly positively correlated with their serum ferritin levels and transferrin saturation (TSAT) levels, but no correlations were found between their serum hepcidin levels and RET-He levels, thereby suggesting that hepcidin has no effect on the iron dynamics of reticulocytes. Since low serum levels of CRP and IL-6, biomarkers of inflammation, were not correlated with the serum hepcidin levels, there is likely to be a threshold for induction of hepcidin expression by inflammation.[30]

In a study by ***MacDougall et al*** PD patients' serum hepcidin levels were significantly positively correlated with their serum ferritin and TSAT levels, but no correlations were found between their serum hepcidin levels and RET-He levels, suggesting that hepcidin has no effect on the iron dynamics of reticulocytes. Stimulation of erythropoiesis by ESA therapy increases the demand for instantly available iron, which often proves insufficient even in patients whose whole-body iron store is not significantly depleted. Absolute iron deficiency in HD patients has been defined based on TSAT and serum ferritin levels, whereas functional iron deficiency results when there is a need for a greater amount of iron to support erythropoiesis than can be supplied. Thus, the conventional methods of estimating iron stores, such as serum ferritin and TSAT measurements, are inadequate to evaluate functional iron deficiency. A strong correlation between serum ferritin and TSAT levels and serum hepcidin levels has been confirmed, but there is no information about the relation between hepcidin and reticulocyte haemoglobin correlation was found between the serum hepcidin levels and reticulocyte haemoglobin levels in this study, suggesting that hepcidin does not directly regulate iron metabolism in newly produced erythrocytes [31]

Biomarkers traditionally used in the diagnosis of IDA include Hb and hematocrit, reticulocyte count, mean corpuscular Hb, and mean corpuscular volume, most of which are decreased in IDA[32] In the setting of absolute iron deficiency, iron studies typically show a decreased iron level, decreased ferritin, elevated transferrin and total iron binding capacity (calculated as transferrin ×1.389), and decreased transferrin saturation (TSAT; calculated as serum iron/total iron binding capacity×100). However, there is evidence to indicate that the traditional cutoffs of TSAT at ≤20% and serum ferritin at ≤100 ng/ml are not sensitive to detect iron deficiency. In a study of 100 patients with CKD (stages 3–5), these indices identified only 17% of patients with CKD as iron deficient whereas approximately 50% were iron deficient based on the gold standard of bone marrow iron staining.[33] Consistent with these findings, patients with iron studies that are within what is considered the normal range or "at goal," may still show an increase in erythropoiesis to trials of iron therapy, whether they are on ESA therapy or not *[33,34]*

Another major limitation of these parameters is that they do not differentiate between absolute and functional IDA. Transferrin, for example, is increased in both absolute and functional IDA. In addition, if functional iron deficiency exists due to a supply/demand mismatch, such as with ESA supplementation, then iron maybe stripped from transferrin faster than it can be mobilized from the iron stores, leading to a decrease in TSAT.10 Bone marrow biopsy is considered by many to be the gold standard for diagnosis of IDA.[35] A study of 303 children in Malawi with iron deficiency concluded that the absence of iron fragments in a sample should be diagnostic of absolute iron deficiency, whereas the absence of erythroid progenitors (despite the presence of iron stores) should be diagnostic of functional iron deficiency. Estimates conclude, however, that up to 30% of bone marrow samples may be inaccurate or insufficient for diagnosis [36] and the number of fragments analyzed greatly affects the yield of a correct diagnosis. Other limitations include the invasiveness of the procedure and the burdens of cost and travel for patients. Given these limitations, there is a need for novel serum biomarkers to differentiate types of IDA in patients with CKD.

LACUNAE IN RESEARCH

There are various studies to demonstrate the role of hepcidin in relation with iron deficiency anemia in CKD, but adequate studies are lacking in association with quantitative measurement of hepcidin and its correlation with different stages of CKD. Despite Hepcidin being a key regulator of iron homeostasis, its study in the setting of chronic kidney disease (CKD) has been hampered by the lack of validated serum assays.

AIMS AND OBJECTIVES

AIM:

- To correlate Hepcidin in Absolute and functional Iron deficiency anemia in chronic kidney disease patients

Primary Objective:

- To correlate Serum Hepcidin levels with Absolute and functional IDA parameters in chronic kidney disease patients.

MATERIALS AND METHODS

PLACE OF STUDY: The study was conducted from January 2021 to June 2022 at Department of Nephrology AIIMS, Jodhpur.

STUDY DESIGN: Observational and Cross-Sectional Study.

SAMPLE SIZE: Time bound study. All Patients fulfilling inclusion criteria between the study periods from January 2021 till June 2022 were included in the study

PATIENT SELECTION

Patients with CKD with iron deficiency were included in the study. Anemia was defined as a Hb concentration <12.0 g/dL in both males and females. The Kidney Disease: Improving Global Outcomes guidelines defines anemia as Hb <13 g/dL in males and <12 g/dL in females [1]. Hb <12.0 g/dL but >10.0 g/dL was defined as mild, Hb <10.0 g/dL but >8.0 g/dL was defined as moderate and severe anemia was defined as Hb <8.0 g/dL. Among CKD patients, absolute iron deficiency was defined when the transferrin saturation (TSAT) is ≤20% and the serum ferritin concentration is ≤100 ng/mL among predialysis and peritoneal dialysis patients or ≤200 ng/mL among hemodialysis patients.[15]. Functional Iron deficiency was defined as TSAT <20% and ferritin 100–500 ng/mL

Inclusion Criteria:

Chronic kidney disease patients with IDA above the age of 18 years.

Exclusion Criteria:

1) Chronic illnesses as Tuberculosis, Malignancy, Retro positive, chronic Liver disease (CLD).
2) Patient not willing to give consent for the study
3) Sepsis
4) Neurological disease like cerebrovascular accident.
5) Pregnancy

METHODS

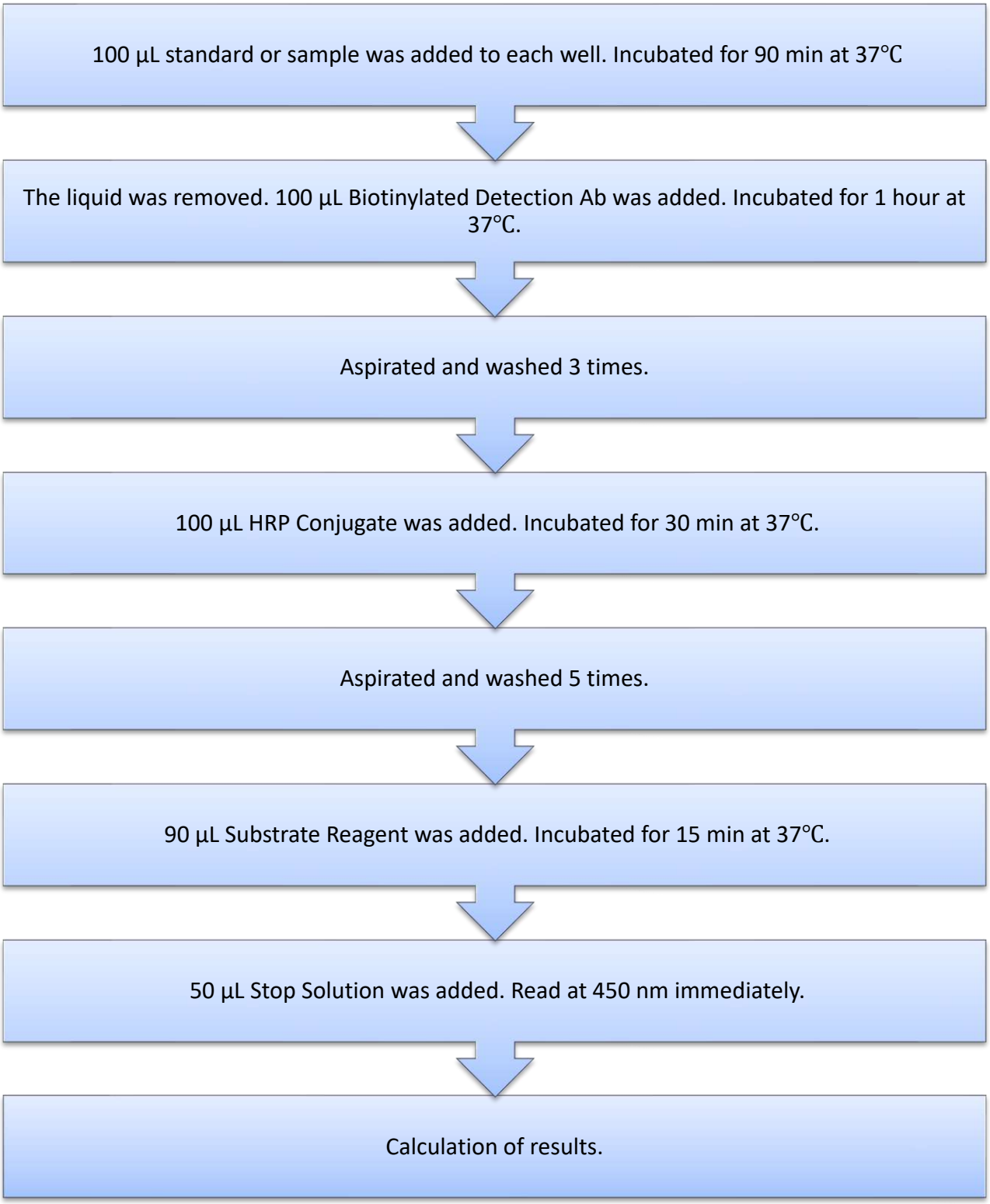
After clearance from the Institute Ethical Committee and patient Informed Consent was obtained, the patients who are CKD as defined by KDIGO guidelines were recruited. Patients were admitted or treated on OPD/IPD basis in the department of Nephrology. The following tests were be done in patients with CKD for diagnosis of Functional IDA, Absolute IDA, and assessment of serum Hepcidin levels.

1) Complete blood count
2) Renal function test/Serum electrolytes.
3) Liver function test including serum albumin
4) Peripheral blood smear/Reticulocyte count
5) Intact PTH/ Vitamin-D/ Calcium/Phosphorus
6) Serum Iron/TIBC/Serum Ferritin/Hepcidin/Ret He
7) Serum Vitamin B12 Levels

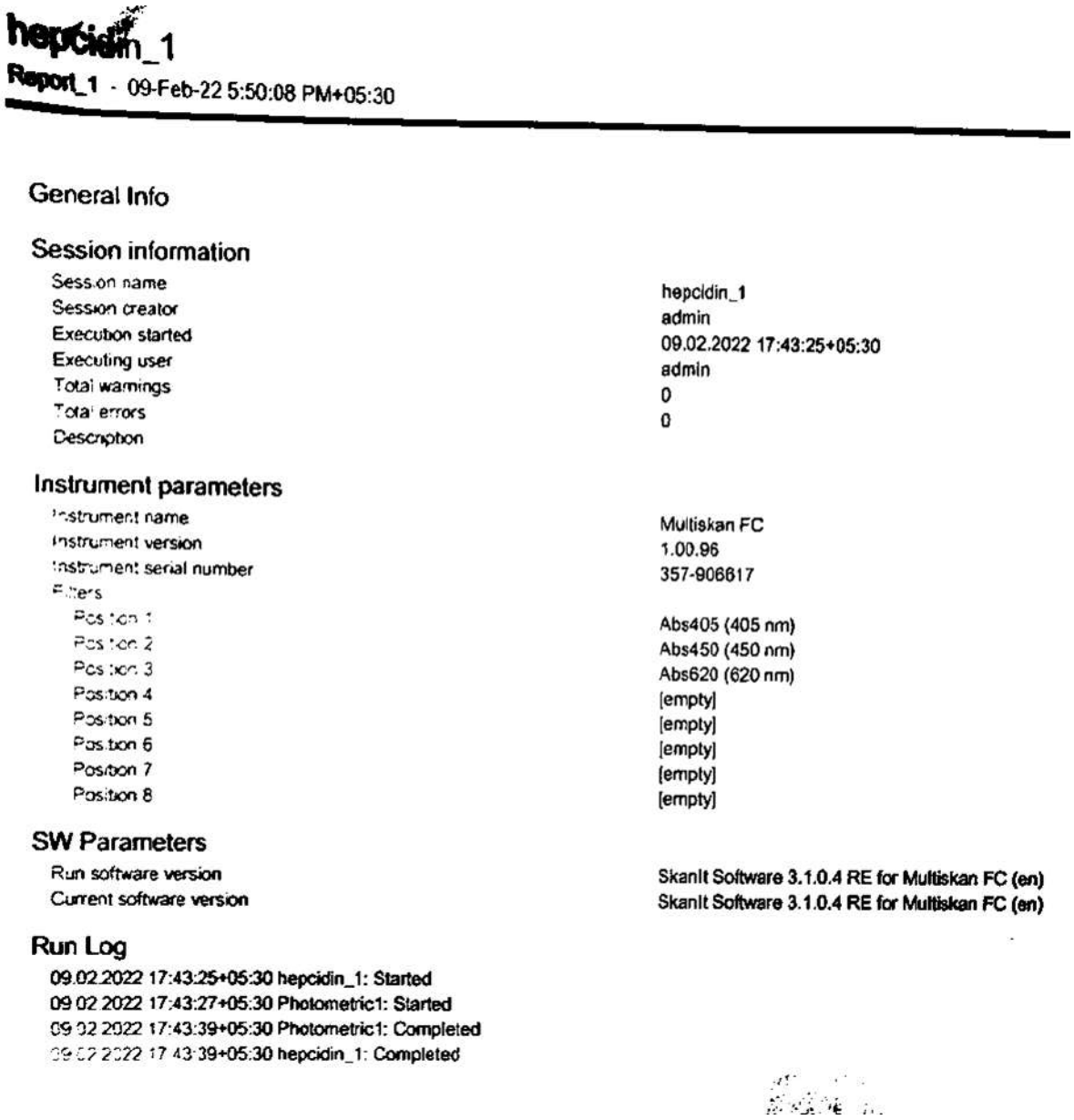
ESTIMATION OF HEPCIDIN (ELISA):

Test principle:

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate pre-coated with an antibody specific to Human Hepcidin was used. Samples (or Standards) were added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human Hepcidin and Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components are washed away. The substrate solution was added to each well. Only those wells that contain Human Hepcidin, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human Hepcidin. We calculated the concentration of Human Hepcidin in the samples by comparing the OD of the samples to the standard curve.


100 µL standard or sample was added to each well. Incubated for 90 min at 37℃
The liquid was removed. 100 µL Biotinylated Detection Ab was added. Incubated for 1 hour at 37℃.
Aspirated and washed 3 times.
100 µL HRP Conjugate was added. Incubated for 30 min at 37℃.
Aspirated and washed 5 times.
90 µL Substrate Reagent was added. Incubated for 15 min at 37℃.
50 µL Stop Solution was added. Read at 450 nm immediately.
Calculation of results.

Picture 2



hepcidin_1

Report_1 - 09-Feb-22 5:50:08 PM+05:30

General Info

Session information

Session name	hepcidin_1
Session creator	admin
Execution started	09.02.2022 17:43:25+05:30
Executing user	admin
Total warnings	0
Total errors	0
Description	

Instrument parameters

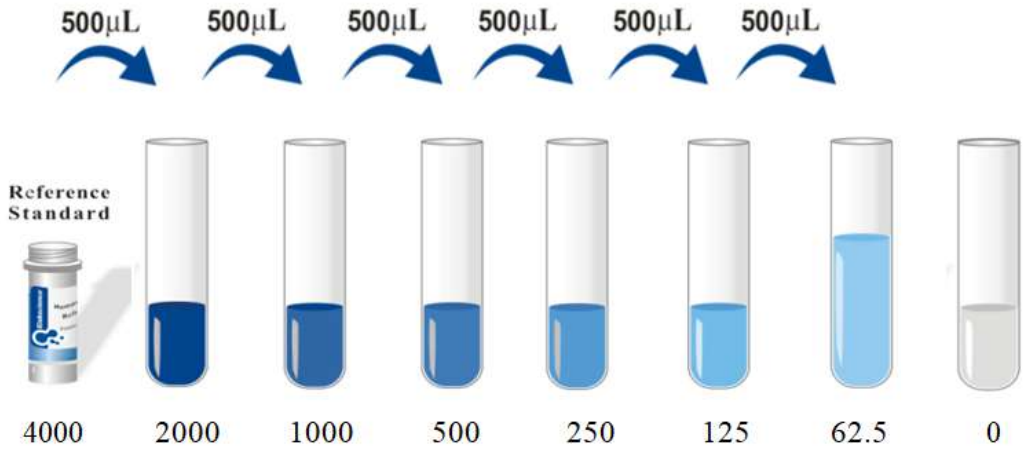
Instrument name	Multiskan FC
Instrument version	1.00.96
Instrument serial number	357-906617
Filters	
Position 1	Abs405 (405 nm)
Position 2	Abs450 (450 nm)
Position 3	Abs620 (620 nm)
Position 4	[empty]
Position 5	[empty]
Position 6	[empty]
Position 7	[empty]
Position 8	[empty]

SW Parameters

Run software version	SkanIt Software 3.1.0.4 RE for Multiskan FC (en)
Current software version	SkanIt Software 3.1.0.4 RE for Multiskan FC (en)

Run Log

09.02.2022 17:43:25+05:30 hepcidin_1: Started
09.02.2022 17:43:27+05:30 Photometric1: Started
09.02.2022 17:43:39+05:30 Photometric1: Completed
09.02.2022 17:43:39+05:30 hepcidin_1: Completed


500µL
500µL
500µL
500µL
500µL
500µL
Reference Standard
4000
2000
1000
500
250
125
62.5
0

Picture 3

QuantitativeCurveFit1

Parameters

Fit to	Assay: Calibrator
Fit type	Four Parameter Logistic
Concentration transform	Linear
Measurement transform	Linear
Markers	Mean
Formula	y = d + (a - d)/(1 + (x / c)^b)
Parameter a	0.0684479145570341
Parameter b	1.13433334403531
Parameter c	3635.39835320446
Parameter d	3.48380720945776
Coefficient of determination R2	0.5847 (Low R2)

Graph

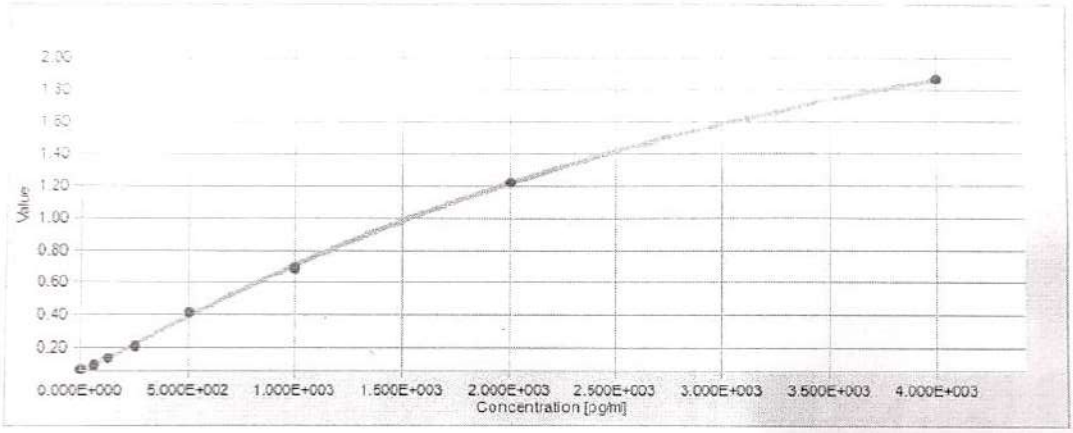


Plate	Well	Sample	Conc.	Original [Abs]	Fitted [Abs]	Residual
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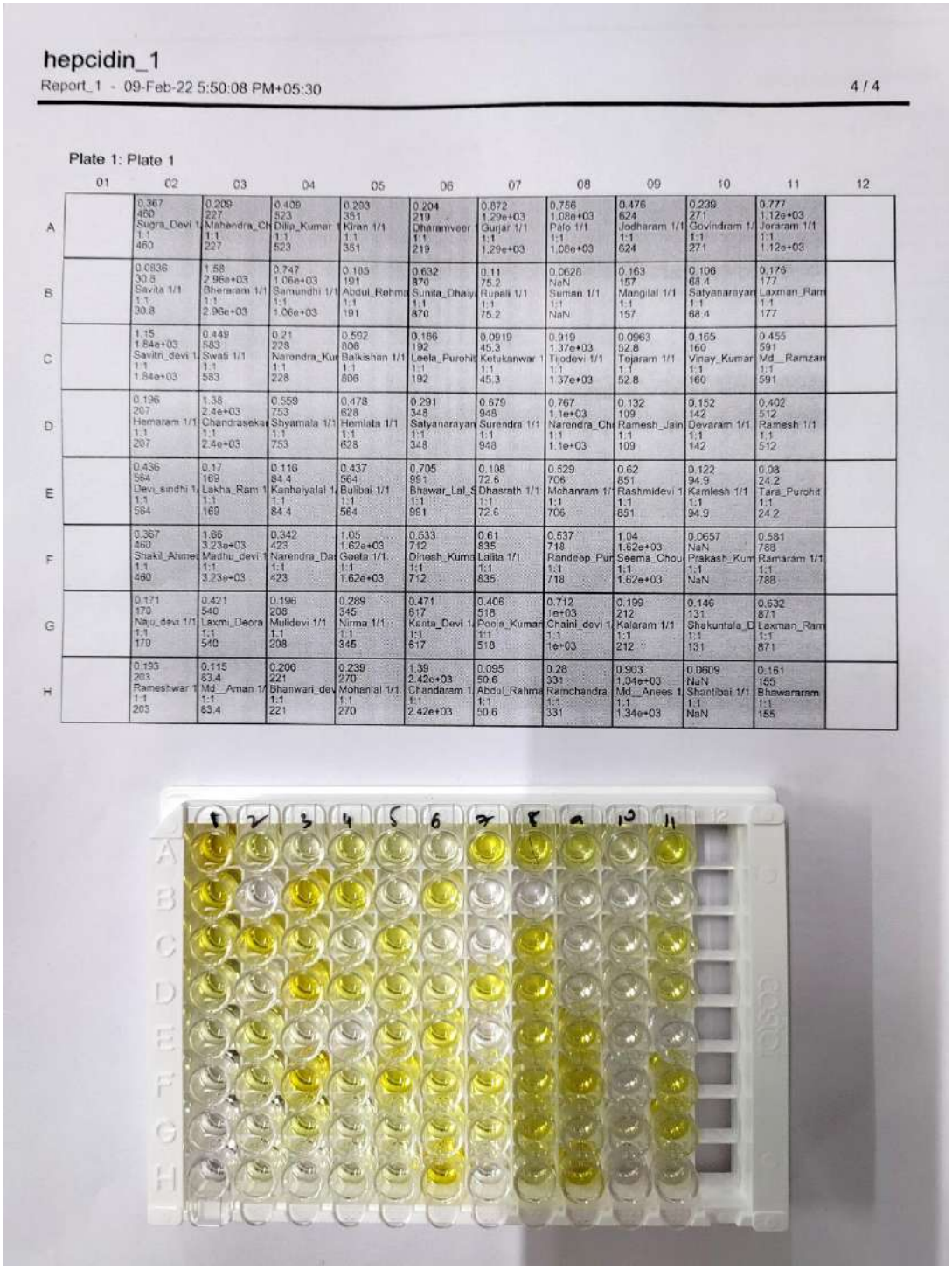
Plate 1	A01	Cal_0001 1/1	4000.00000	1.87	1.87	-0.001
		Cal_0002	2000.00000	1.22	1.22	0.006
Plate 1	B01	Cal_0002 1/1	2000.00000	1.22	1.22	0.006
		Cal_0003	1000.00000	0.693	0.71	-0.017
Plate 1	C01	Cal_0003 1/1	1000.00000	0.693	0.71	-0.017
		Cal_0004	500.00000	0.42	0.394	0.026
Plate 1	D01	Cal_0004 1/1	500.00000	0.42	0.394	0.026
		Cal_0005	250.00000	0.208	0.225	-0.017
Plate 1	E01	Cal_0005 1/1	250.00000	0.208	0.225	-0.017
		Cal_0006	125.00000	0.144	0.142	0.002
Plate 1	F01	Cal_0006 1/1	125.00000	0.144	0.142	0.002
		Cal_0007	62.50000	0.1	0.102	-0.002
Plate 1	G01	Cal_0007 1/1	62.50000	0.1	0.102	-0.002
		Cal_0008	0	0.0711	0.0684	0.003
Plate 1	H01	Cal_0008 1/1	0	0.0711	0.0684	0.003

Picture 4



hepcidin_1

Report_1 - 09-Feb-22 5:50:08 PM+05:30 4 / 4

Plate 1: Plate 1

	01	02	03	04	05	06	07	08	09	10	11	12
A		0.367 460 Sugra_Devi 1 1:1 460	0.209 227 Mahendra_Ch 1:1 227	0.409 523 Dilip_Kumar 1 1:1 523	0.293 351 Kiran 1/1 1:1 351	0.204 219 Dharamveer 1:1 219	0.872 1.29e+03 Gurjar 1/1 1:1 1.29e+03	0.756 1.08e+03 Palo 1/1 1:1 1.08e+03	0.476 624 Jodharam 1/1 1:1 624	0.239 271 Govindram 1/ 1:1 271	0.777 1.12e+03 Joraram 1/1 1:1 1.12e+03	
B		0.0836 30.8 Savita 1/1 1:1 30.8	1.58 2.96e+03 Bheraram 1/1 1:1 2.96e+03	0.747 1.06e+03 Samundhi 1/1 1:1 1.06e+03	0.105 191 Abdul_Rehma 1:1 191	0.632 870 Sunita_Dhaiy 1:1 870	0.11 75.2 Rupali 1/1 1:1 75.2	0.0628 NaN Suman 1/1 1:1 NaN	0.163 157 Mangilal 1/1 1:1 157	0.106 68.4 Satyanarayan 1:1 68.4	0.176 177 Laxman_Ram 1:1 177	
C		1.15 1.84e+03 Savitri_devi 1 1:1 1.84e+03	0.449 583 Swati 1/1 1:1 583	0.21 228 Narendra_Kur 1:1 228	0.592 806 Balkishan 1/1 1:1 806	0.186 192 Leela_Purohit 1:1 192	0.0919 45.3 Ketukanwar 1 1:1 45.3	0.919 1.37e+03 Tijodevi 1/1 1:1 1.37e+03	0.0963 52.8 Tejaram 1/1 1:1 52.8	0.165 160 Vinay_Kumar 1:1 160	0.455 591 Md__Ramzan 1:1 591	
D		0.196 207 Hemaram 1/1 1:1 207	1.38 2.4e+03 Chandrasekar 1:1 2.4e+03	0.559 753 Shyamala 1/1 1:1 753	0.478 628 Hemlata 1/1 1:1 628	0.291 348 Satyanarayan 1:1 348	0.679 948 Surendra 1/1 1:1 948	0.767 1.1e+03 Narendra_Ch 1:1 1.1e+03	0.132 109 Ramesh_Jain 1:1 109	0.152 142 Devaram 1/1 1:1 142	0.402 512 Ramesh 1/1 1:1 512	
E		0.436 564 Devi_sindhi 1 1:1 564	0.17 169 Lakha_Ram 1 1:1 169	0.116 84.4 Kanhaiyalal 1 1:1 84.4	0.437 564 Bulibai 1/1 1:1 564	0.705 991 Bhawar_Lal_S 1:1 991	0.108 72.6 Dhasrath 1/1 1:1 72.6	0.529 706 Mohanram 1/ 1:1 706	0.62 851 Rashmidevi 1 1:1 851	0.122 94.9 Kamlesh 1/1 1:1 94.9	0.08 24.2 Tara_Purohit 1:1 24.2	
F		0.367 460 Shakil_Ahmed 1:1 460	1.86 3.23e+03 Madhu_devi 1 1:1 3.23e+03	0.342 423 Narendra_Da 1:1 423	1.05 1.62e+03 Geeta 1/1 1:1 1.62e+03	0.533 712 Dinesh_Kuma 1:1 712	0.61 835 Lalita 1/1 1:1 835	0.537 718 Randeep_Pur 1:1 718	1.04 1.62e+03 Seema_Chou 1:1 1.62e+03	0.0657 NaN Prakash_Kum 1:1 NaN	0.581 788 Ramaram 1/1 1:1 788	
G		0.171 170 Naju_devi 1/1 1:1 170	0.421 540 Laxmi_Deora 1:1 540	0.196 208 Mulidevi 1/1 1:1 208	0.289 345 Nirma 1/1 1:1 345	0.471 617 Kanta_Devi 1 1:1 617	0.406 518 Pooja_Kumari 1:1 518	0.712 1e+03 Chaini_devi 1 1:1 1e+03	0.199 212 Kalaram 1/1 1:1 212	0.146 131 Shakuntala_D 1:1 131	0.632 871 Laxman_Ram 1:1 871	
H		0.193 203 Rameshwar 1 1:1 203	0.115 83.4 Md__Aman 1/ 1:1 83.4	0.206 221 Bhanwari_dev 1:1 221	0.239 270 Mohanlal 1/1 1:1 270	1.39 2.42e+03 Chandaram 1 1:1 2.42e+03	0.095 50.6 Abdul_Rahma 1:1 50.6	0.28 331 Ramchandra 1:1 331	0.903 1.34e+03 Md__Anees 1 1:1 1.34e+03	0.0609 NaN Shantibai 1/1 1:1 NaN	0.151 155 Bhawararam 1:1 155	

DATA COLLECTION

Data was collected on the first visit for the baseline assessment using following:

1. Proforma (ANNEXURE 5)

STATISTICAL ANALYSIS

Data was entered in the excel spread sheet. **SPSS (Statistical Package for Social Sciences)** version 20. [IBM SPSS statistics was used to perform the statistical analysis. Descriptive statistics of the explanatory and outcome variables were calculated by mean, standard deviation for quantitative variables, frequency, and proportions for qualitative variables. Chi square was applied to test the statistical association between qualitative variables. ANOVA test was applied to test the statistical significance for more than two groups for quantitative data. Pearson's correlation was calculated, and scatter plots were drawn to calculate the correlation between quantitative variables. The level of significance was set at 5%.

ETHICAL CONSIDERATION

The purpose of the present study was to assess Serum Hepcidin in patients with Absolute and Functional IDA in CKD patients at AIIMS Jodhpur. The clinical significance of Hepcidin and its role in iron deficiency anemia in different stages of CKD has already been established in human studies. All tests were done after informing patients and their attendants that they are a part of the study and after taking full informed consent. There were no potential risks for the patients participating in the study.

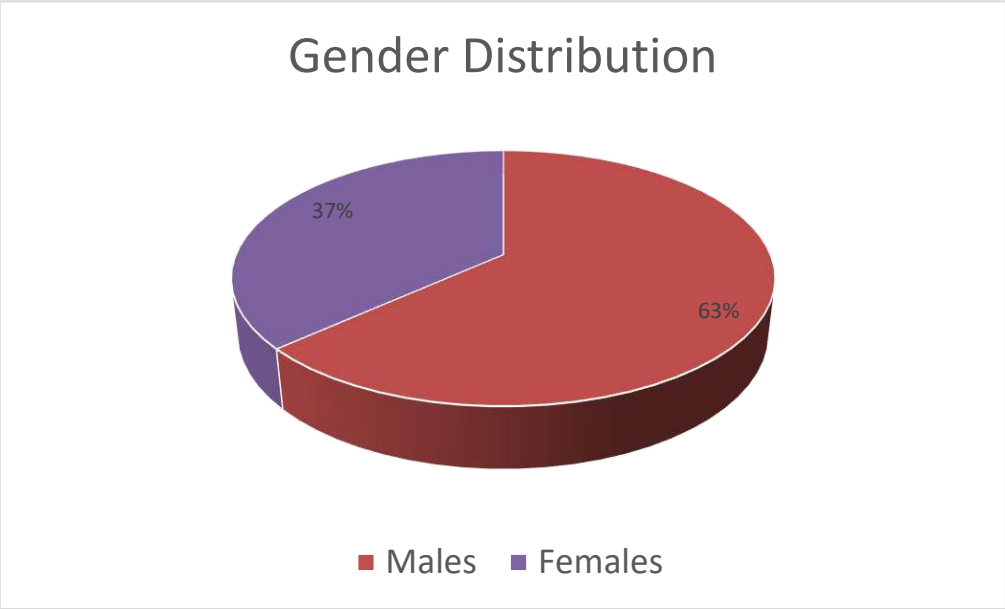
RESULTS

Demographic data

Table 1: Age distribution of the study group

Variables	CKD stage	N	Mean	\Std. Dev
AGE	II	7	39.43	13.024
	IIIa	13	42.92	17.562
	IIIB	18	45.28	15.803
	IV	41	47.73	17.751
	V	60	41.07	16.874
	Total	139	43.67	16.946

Figure 1: Pie chart of gender distribution



In our study, 139 patients were enrolled out of which 88 (70.5%) were males while the rest 51(36.65%) were females. Majority of patients were in CKD stage V (60) where the mean age was 41 years, stage IIIA, IIIB, IV had 13,18 and 41 patients respectively the average age of patients in our study was 43 years with a standard deviation of 19.9 (Table 1)

Table 2: Urea, creatinine levels and creatinine clearance in different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
UREA	II	7	65.14	24.600	42.39	87.89	10.412	**<0.001***
	IIIa	13	57.08	10.996	50.43	63.72		
	IIIB	18	108.61	183.081	17.57	199.66		
	IV	41	93.56	28.950	84.42	102.70		
	V	60	168.53	54.510	154.45	182.61		
	Total	139	123.03	86.281	108.56	137.50		
Cr	II	7	1.71	.951	.83	2.59	63.574	**<0.001***
	IIIa	13	1.85	.376	1.62	2.07		
	IIIB	18	2.44	.511	2.19	2.70		
	IV	41	3.90	1.136	3.54	4.26		
	V	60	8.60	2.993	7.83	9.37		
	Total	139	5.44	3.524	4.85	6.03		
CrCl ml/min/m^2	II	7	53.86	15.137	39.86	67.86	256.763	**<0.001***
	IIIa	13	50.85	4.598	48.07	53.62		
	IIIB	18	36.61	4.754	34.25	38.98		
	IV	41	20.90	4.862	19.37	22.44		
	V	60	9.60	4.291	8.49	10.71		
	Total	139	22.52	15.936	19.85	25.19		

*- statistically significant by ANOVA test

As seen in data from table 2, the values of urea and creatinine increase with increasing stages of CKD along with a fall in creatinine clearance, the average levels of urea and creatinine in stage V were 168 mg and 8.60 mg respectively with a standard deviation of 54.5 and 2.99. Mean creatinine clearance in stage V was 9.60 ml/min/m^2.

Table 3: Levels of ESR and CRP in different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
ESR	II	7	38.57	23.165	17.15	60.00	6.634	**<0.001***
	IIIa	13	42.08	24.649	27.18	56.97		
	IIIB	18	38.17	16.635	29.89	46.44		
	IV	41	58.05	26.628	49.64	66.45		
	V	60	64.57	23.244	58.56	70.57		
	Total	139	55.81	25.594	51.52	60.11		
CRP	II	7	23.00	15.011	9.12	36.88	2.297	0.062
	IIIa	13	25.69	31.351	6.75	44.64		
	IIIB	18	50.28	37.910	31.43	69.13		
	IV	40	51.23	45.392	36.71	65.74		
	V	60	60.02	50.884	46.87	73.16		
	Total	138	51.09	45.961	43.35	58.82		

*- statistically significant by ANOVA test

Chronic kidney disease is an inflammatory state and with worsening creatinine clearance, levels of inflammatory markers such as ESR and CRP were also found higher in CKD stage V in our study, the mean levels of ESR was 64.57 with a standard deviation of 23.24, mean CRP levels in stage V CKD were 60.02 with a standard deviation of 50.884. ESR levels showed statistical significance with advancing CKD as shown by p values of <0.001 by ANOVA test. (Table 4)

Figure 2: Graphical representation of ESR and CRP across various stages of CKD

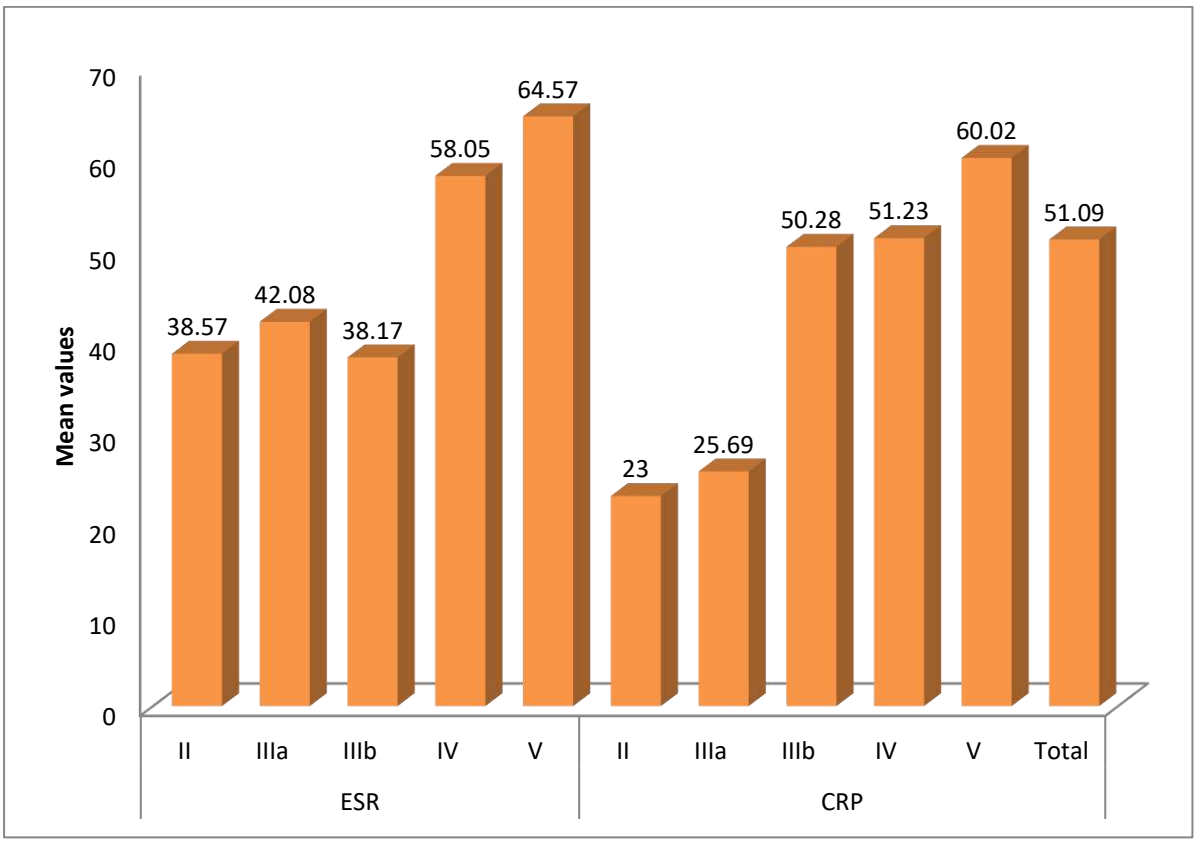


Table 4: Calcium and phosphorous levels in different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
Ca	II	7	8.17	.898	7.34	9.00	1.509	0.203
	IIIa	13	8.49	.930	7.93	9.05		
	IIIB	18	8.27	0.829	7.86	8.68		
	IV	41	8.10	.995	7.78	8.41		
	V	60	7.82	1.168	7.52	8.13		
	Total	139	61.08	625.197	-43.78	165.93		
PHOSP	II	7	5.14	2.035	3.26	7.03	2.622	0.038
	IIIa	13	5.77	1.964	4.58	6.96		
	IIIB	18	5.33	1.414	4.63	6.04		
	IV	41	5.10	1.670	4.57	5.62		
	V	60	6.30	2.287	5.71	6.89		
	Total	139	5.71	2.026	5.37	6.05		

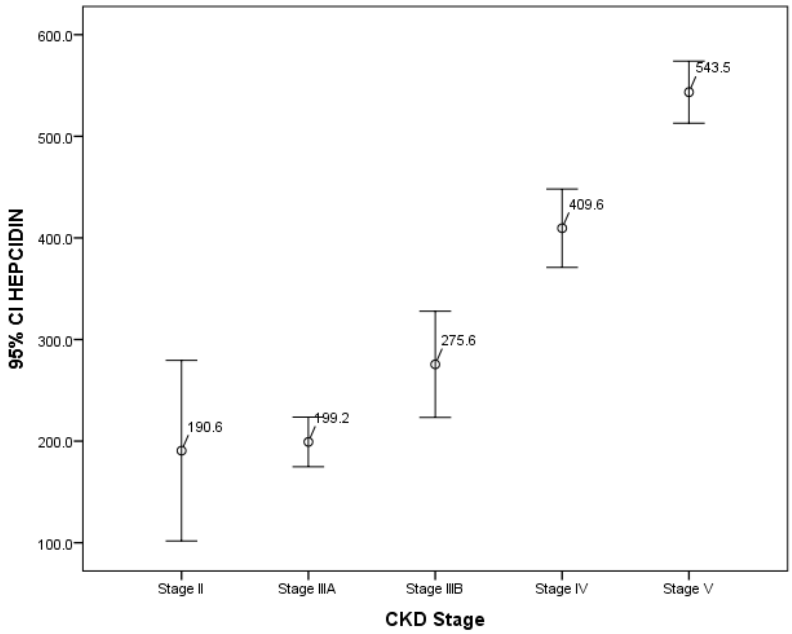
Mean serum calcium levels in CKD stage II was 8.17, in stage IIIa was 8.49, stage IIIB was 8.27, stage IV was 8.10 and in stage V was 7.82. The difference in mean serum calcium levels in various stages of CKD was not statistically significant by ANOVA test. Mean serum phosphorous levels in CKD stage II was 5.14, in stage IIIa was 5.77, stage IIIB was 5.33, stage IV was 5.10 and in stage V was 6.30. The difference in mean serum phosphorous levels in various stages of CKD was not statistically significant by ANOVA test. (Table 5)

Table 5: Comparison of Hepcidin levels in different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
HEPCIDIN	II	7	190.57	96.095	101.70	279.44	45.602	<0.001*
	IIIa	13	199.23	40.493	174.76	223.70		
	IIIB	18	275.56	104.994	223.34	327.77		
	IV	41	409.56	122.138	371.01	448.11		
	V	60	543.47	118.462	512.86	574.07		
	Total	139	419.31	169.907	390.81	447.80		

*- statistically significant by ANOVA test

Figure 3: Distribution of mean Hepcidin levels across various stages of CKD



Using One-way ANOVA, we found that hepcidin levels were significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were found between all the groups except between stages II, IIIA and IIIB .

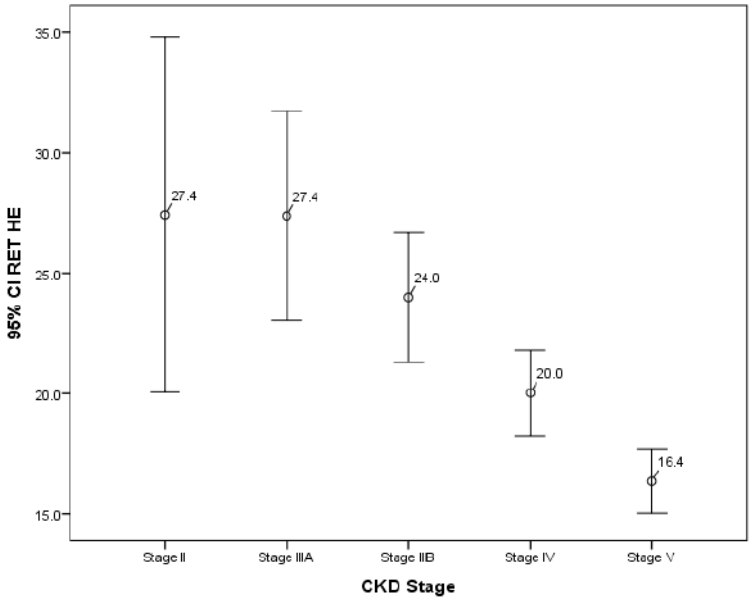
Mean hepcidin levels in CKD stage II was 190.57, in stage IIIa was 199.23, stage IIIB was 275.56, stage IV was 409.56 and in stage V was 543.47. The increase in mean hepcidin levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$). (Table 6)

Table 6: Comparison of RET HE levels across different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
RET HE	II	7	27.43	7.976	20.05	34.81	16.919	**<0.001***
	IIIa	13	27.38	7.183	23.04	31.73		
	IIIB	18	24.00	5.402	21.31	26.69		
	IV	41	20.02	5.646	18.24	21.81		
	V	60	16.35	5.125	15.03	17.67		
	Total	139	20.01	6.865	18.86	21.17		

*- statistically significant by ANOVA test

Figure 4: Distribution of mean Ret HE levels across various stages of CKD



Using One-way ANOVA, we found that Ret HE levels were significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were found between IIIB , IV and V.

Mean RET HE levels in CKD stage II was 27.43, in stage IIIa was 27.38, stage IIIB was 24, stage IV was 20.02 and in stage V was 16.35. The decrease in mean RET HE levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$). (Table 7)

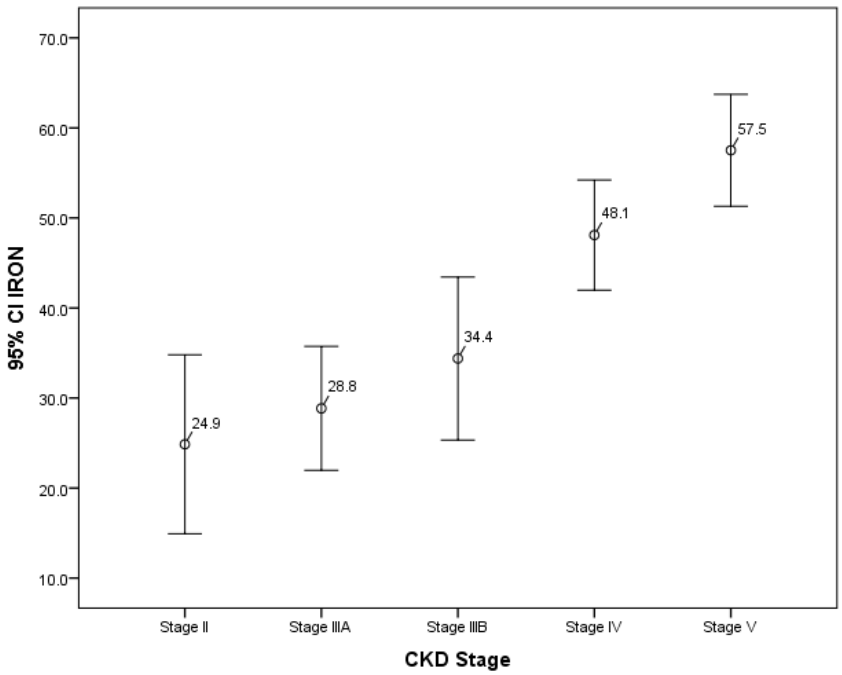
Table 7: Comparison of Iron Deficiency anemia parameters different stages of chronic kidney disease.

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
IRON	II	7	24.86	10.746	14.92	34.80	10.125	**<0.001***
	IIIa	13	28.85	11.393	21.96	35.73		
	IIIB	18	34.39	18.199	25.34	43.44		
	IV	41	48.10	19.393	41.98	54.22		
	V	60	57.52	24.047	51.30	63.73		
	Total	139	47.42	23.201	43.53	51.31		
TIBC	II	7	208.57	69.173	144.60	272.55	0.511	0.728
	IIIa	13	201.08	49.116	171.40	230.76		
	IIIB	18	192.72	54.198	165.77	219.67		
	IV	41	212.63	64.212	192.37	232.90		
	V	60	212.98	57.022	198.25	227.71		
	Total	139	208.92	58.448	199.12	218.72		
TSAT %	II	7	12.00	3.215	9.03	14.97	12.104	**<0.001***
	IIIa	13	14.31	4.715	11.46	17.16		
	IIIB	18	17.50	8.501	13.27	21.73		
	IV	41	24.15	10.061	20.97	27.32		
	V	60	27.65	8.902	25.35	29.95		
	Total	139	23.27	10.071	21.58	24.96		
FERRITIN	II	7	249.14	118.943	139.14	359.15	20.369	**<0.001***
	IIIa	13	303.92	67.233	263.29	344.55		
	IIIB	18	346.50	194.196	249.93	443.07		
	IV	41	603.51	258.820	521.82	685.21		
	V	60	913.92	425.479	804.00	1023.83		
	Total	139	658.35	405.921	590.27	726.43		

*- statistically significant by ANOVA test

Mean ferritin levels in CKD stage II was 249.14, in stage IIIa was 303.92, stage IIIB was 346.50, stage IV was 603.51 and in stage V was 913.92. The increase in mean ferritin levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$). (Table 8)

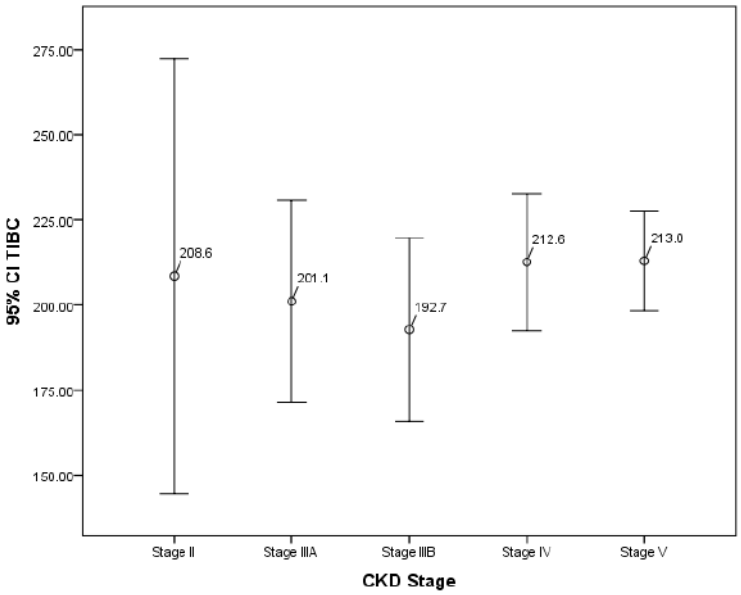
Figure 5: Distribution of mean Iron levels across various stages of CKD



Using One-way ANOVA, we found that Iron levels were significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were found between all the groups except between stages II, IIIA and IIIB

Mean iron levels in CKD stage II were 24.86, in stage IIIa was 28.85, stage IIIB was 34.39, stage IV was 48.10 and in stage V was 57.52. The increase in mean iron levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$).

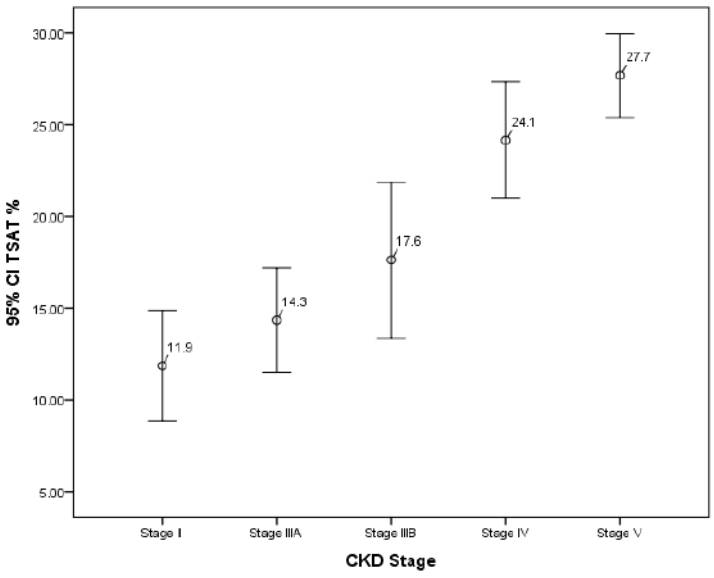
Figure 6: Distribution of mean TIBC levels across various stages of CKD



Using One-way ANOVA, we found that TIBC levels were not significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were not found between any groups.

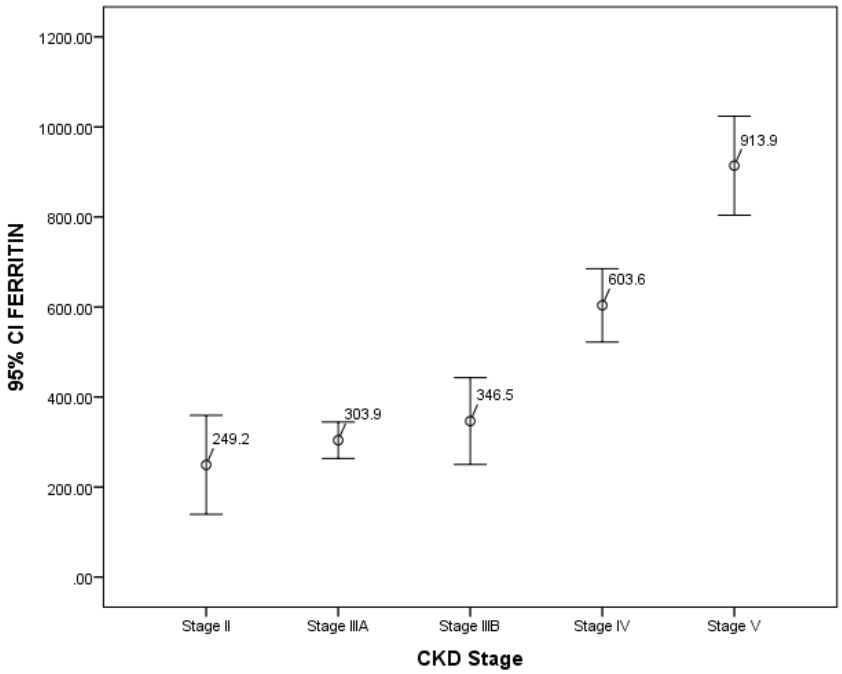
Mean TIBC in CKD stage II was 208.57, in stage IIIa was 201.08, stage IIIB was 192.72, stage IV was 212.63 and in stage V was 212.98. The difference in TIBC levels in various stages of CKD was not statistically significant by ANOVA test.

Figure 7: Distribution of mean TSAT levels across various stages of CKD



Using One-way ANOVA, we found that TSAT levels were significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were found between all the groups except between stages II, IIIA and IIIB .

Figure 8: Distribution of mean Ferritin levels across various stages of CKD



Using One-way ANOVA, we found that Ferritin levels were significantly different across various stages of CKD. On Post-hoc Bonferroni test, significant differences were found between all the groups except between stages II, IIIA and IIIB.

Table 8: Vitamin D and Intact PTH levels in different stages of CKD

Variables	CKD stage	N	Mean	Std. Dev	95% CI for Mean		F value	p value
					Lower Bound	Upper Bound		
VIT D	II	7	31.14	5.900	25.69	36.60	4.308	**0.003***
	IIIa	13	32.54	10.485	26.20	38.87		
	IIIB	18	28.94	15.260	21.36	36.53		
	IV	41	21.78	12.916	17.70	25.86		
	V	60	16.33	7.843	14.31	18.36		
	Total	139	21.83	12.221	19.78	23.88		
PTH pg/ml	II	7	175.57	90.506	91.87	259.28	9.460	**<0.001***
	IIIa	13	339.31	321.166	145.23	533.39		
	IIIB	18	347.61	283.395	206.68	488.54		
	IV	40	498.18	263.436	413.92	582.43		
	V	60	706.27	371.991	610.17	802.36		
	Total	138	537.68	356.078	477.74	597.62		

*- statistically significant by ANOVA test

Mean vitamin D levels in CKD stage II was 31.14, in stage IIIa was 32.54, stage IIIB was 28.94, stage IV was 21.78 and in stage V was 16.33. The decrease in vitamin D levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$).

Mean PTH levels in CKD stage II was 175.57, in stage IIIa was 339.31, stage IIIB was 347.61, stage IV was 498.18 and in stage V was 706.27. The increase in PTH levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$). (Table 9).

Figure 9: Mean PTH levels across different stages of CKD

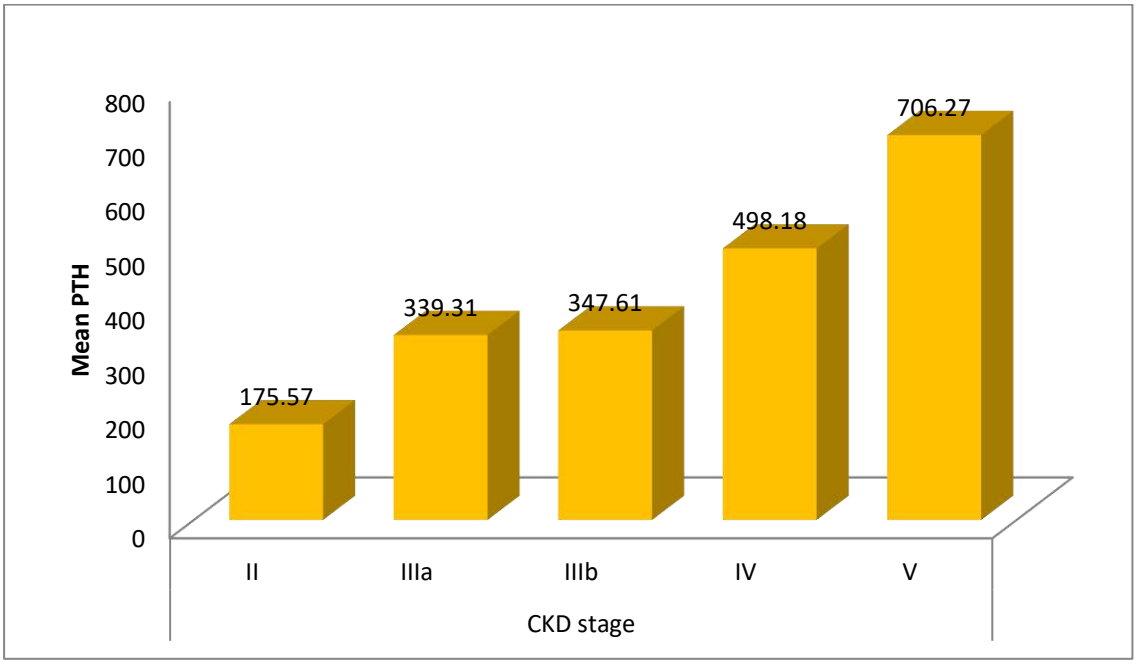


Figure 10 Mean 25-Hydroxy vitamin D levels in different stages of CKD

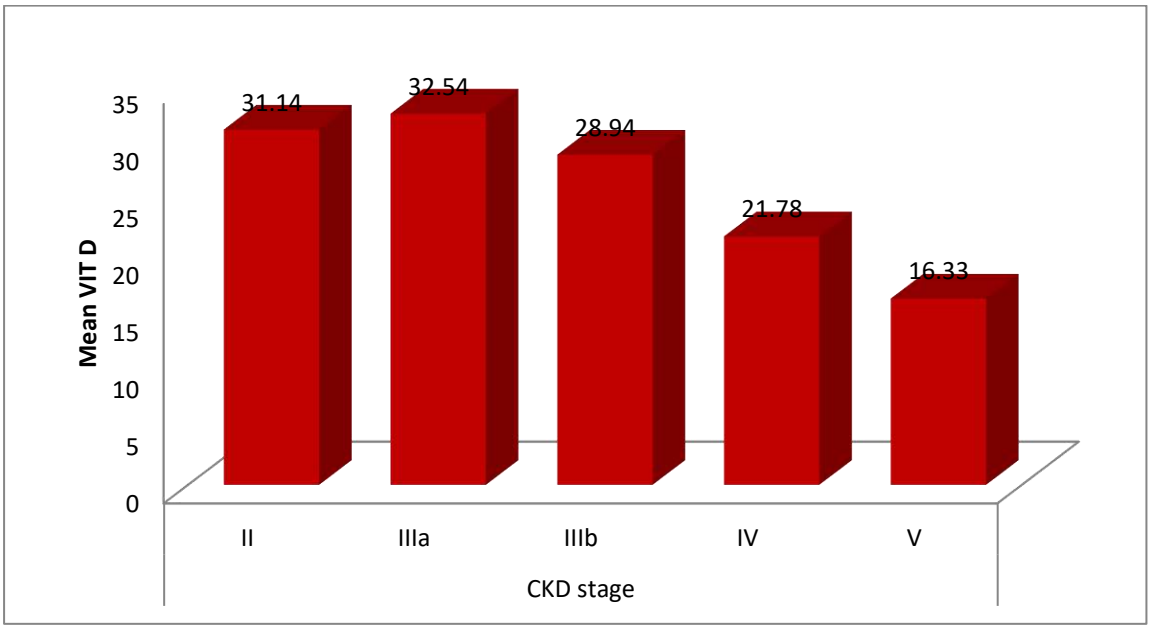


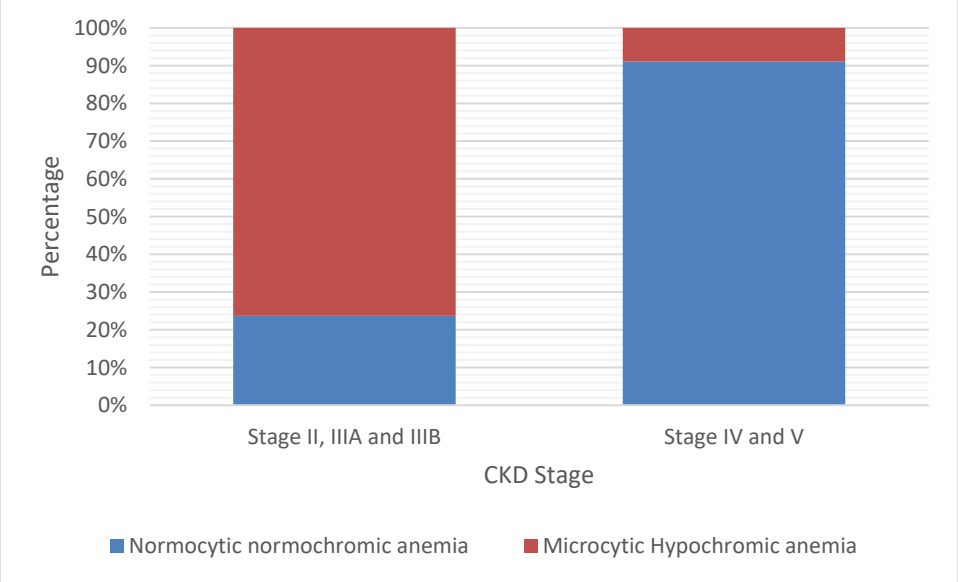
Table 09: Blood film morphology picture with type of anemia in different stages of CKD

		CKD Stage			
		Stage II, IIIA and IIIB		Stage IV and V	
		Count	Column N %	Count	Column N %
Peripheral Smear	Normocytic normochromic anemia	9	23.7%	92	91.1%
	Microcytic Hypochromic anemia	29	76.3%	9	8.9%

P< 0.001, df=1, Chi Square =63.154

23.7 % of patients in early CKD were found to have Normocytic normochromic anemia in comparison to 91.1 % in stages IV and V. 76.3 % were found to have Microcytic hypochromic anemia in CKD II, IIIA AND IIIB while 8.9 % in late stages of CKD

Figure 11 Blood film Morphology in different stages of CKD



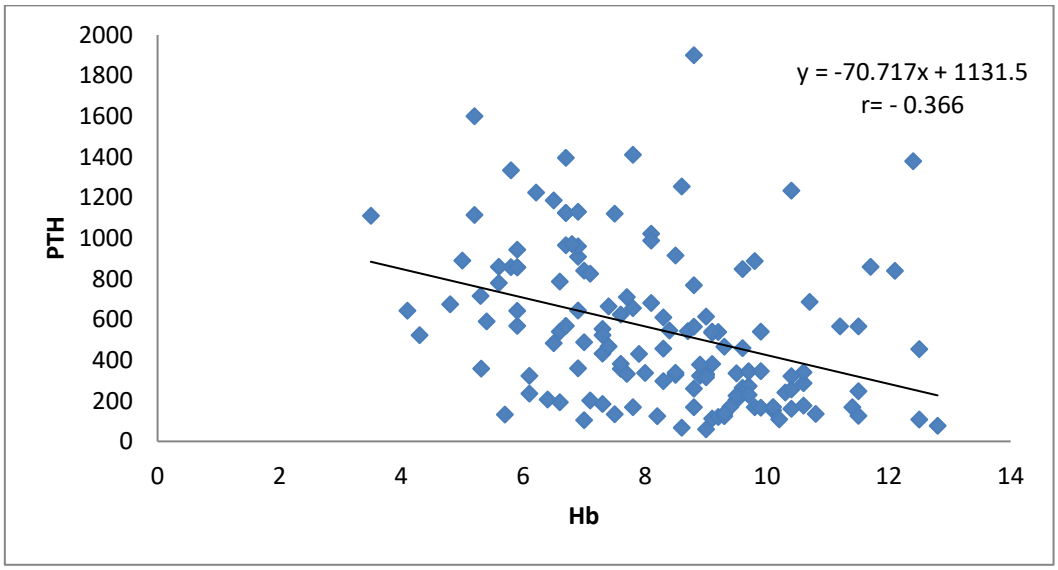
This difference in peripheral smear examination among various stages of CKD was statistically significant by Chi square test ($p < 0.05$)

Table 10: Comparison of various parameters in correlation with Hemoglobin

Pearson correlation with Hb						
Variables	**CKD**	**Stage II**	**Stage IIIA**	**Stage IIIB**	**Stage IV**	**Stage V**
PTH pg/ml	**r value**	.093	-.075	.231	-.115	.019
	p value	.843	.808	.355	.475	.887
	N	7	13	18	41	59
ESR	**r value**	-.674	.056	-.062	0.09	.002
	p value	.097	.856	.807	.576	.990
	N	7	13	18	41	59
CRP	**r value**	-.295	-.202	.365	-.069	**-.258**
	p value	.521	.508	.136	.671	**.048***
	N	7	13	18	40	**59**
Ca	**r value**	**.785**	.401	-.242	.095	.159
	p value	**.037***	.174	.333	.553	.228
	N	**7**	13	18	41	59
FERRITIN	**r value**	-.585	.221	**-.701**	**-.425**	**-.376**
	p value	.168	.468	**.001***	**.006***	**.003***
	N	7	13	**18**	**41**	**59**
PHOSP	**r value**	.383	-.356	.081	.190	**.261**
	p value	.396	.233	.750	.235	**.046***
	N	7	13	18	41	**59**
Hepcidin	**r value**	-.581	.068	**-.561**	**-.364**	**-.386**
	p value	.172	.826	**.015***	**.019***	**.003***
	N	7	13	**18**	**41**	**59**
RET HE	**r value**	.048	-.036	-.085	.237	.253
	p value	.919	.908	.738	.136	.053
	N	7	13	18	41	59

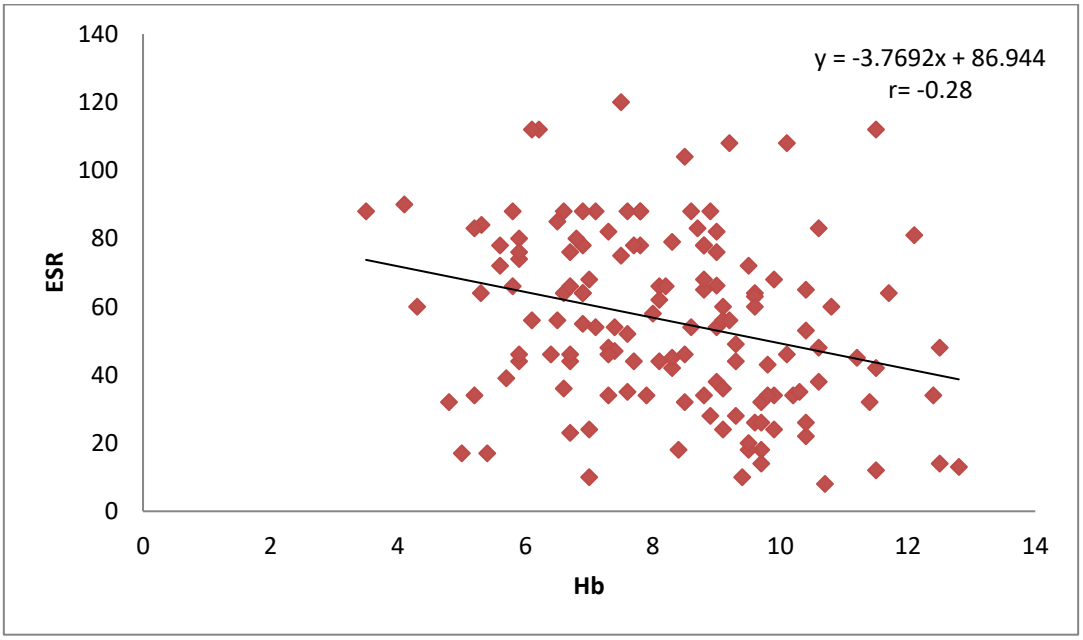
*- statistically significant by Pearson's correlation coefficient

Figure 12 Scatter plot comparison of Intact PTH and Hemoglobin



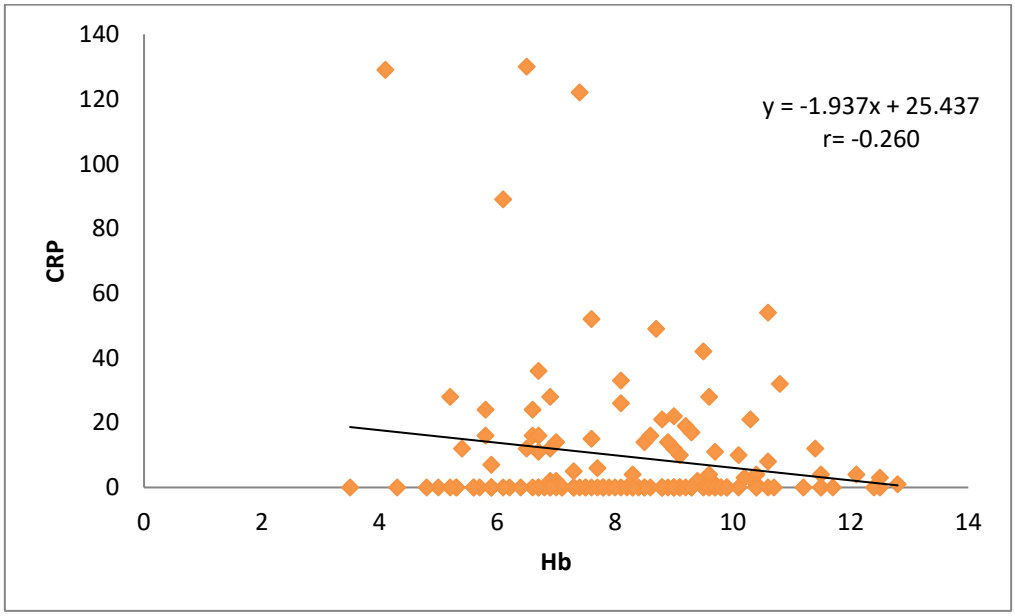
There was no statistically significant correlation between PTH and Hb in various stages of CKD.

Figure 13 Scatter plot comparison of ESR and Hemoglobin



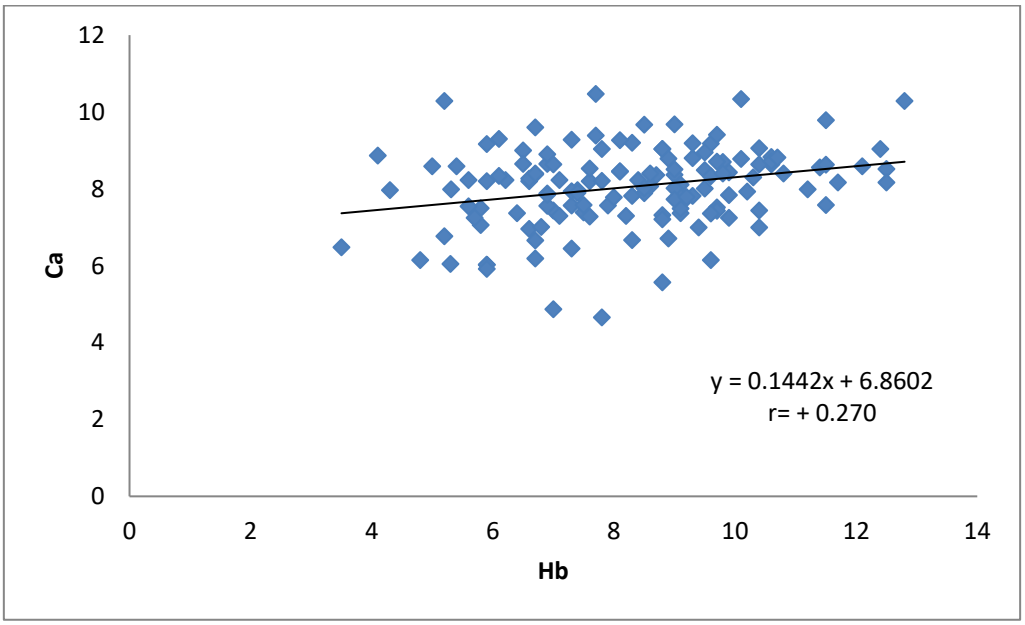
There was no statistically significant correlation between ESR and Hb in various stages of CKD.

Figure 14 Scatter plot comparison of CRP and Hemoglobin



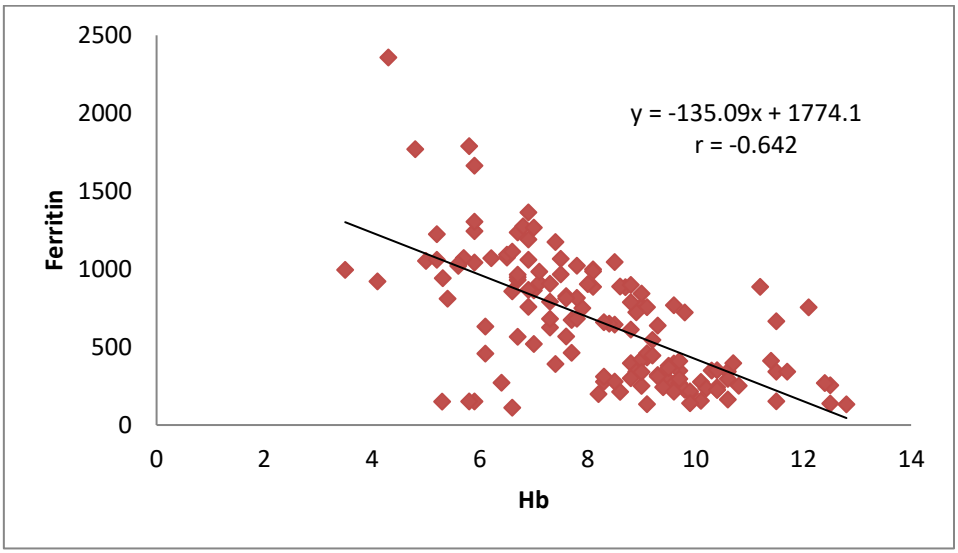
There was a statistically significant negative correlation between CRP and Hb in stage V of CKD.

Figure 15 Scatter plot comparison of Calcium and Hemoglobin



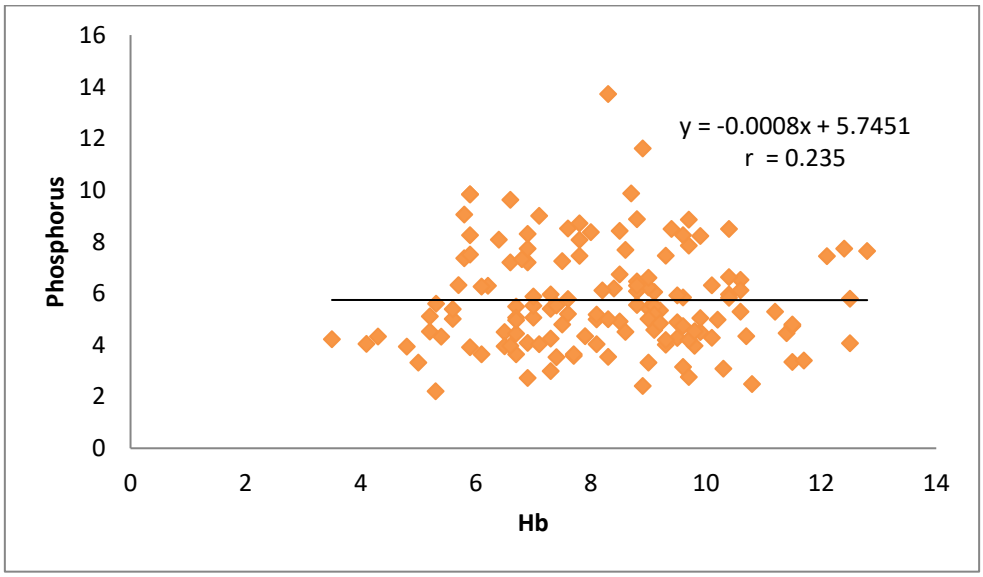
There was a statistically significant strong positive correlation between calcium levels and Hb in stage II of CKD.

Figure 16: Scatter plot comparison of Ferritin and Hemoglobin



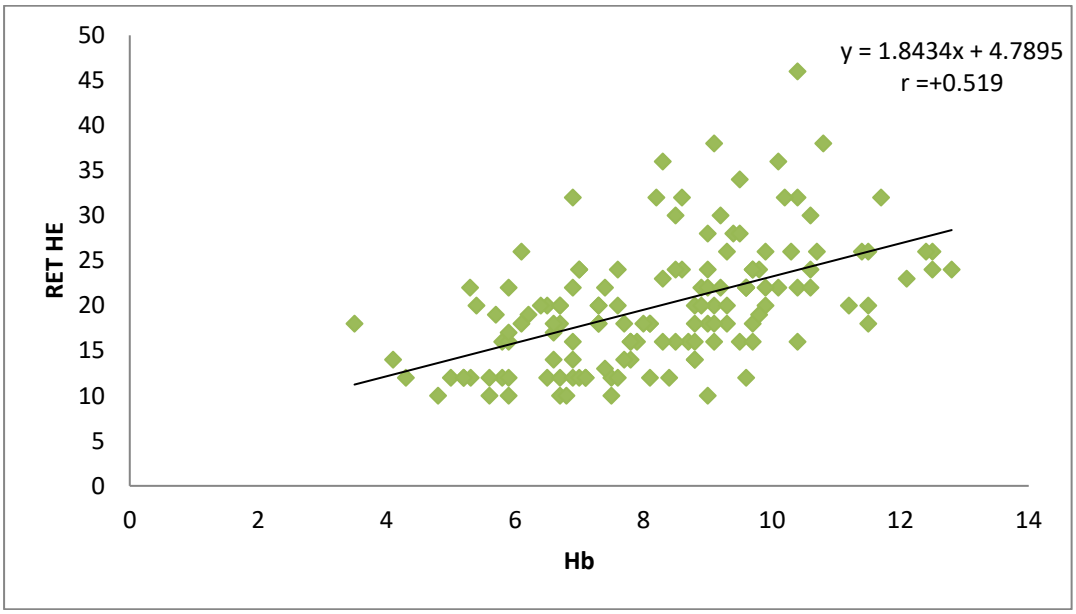
There was a statistically significant strong negative correlation between ferritin levels and Hb in stage IIIb of CKD. There was a statistically significant moderate negative correlation between ferritin levels and Hb in stage IV of CKD. There was a statistically significant negative correlation between ferritin levels and Hb in stage V of CKD

Figure 17: Scatter plot comparison of Phosphorous and Hemoglobin



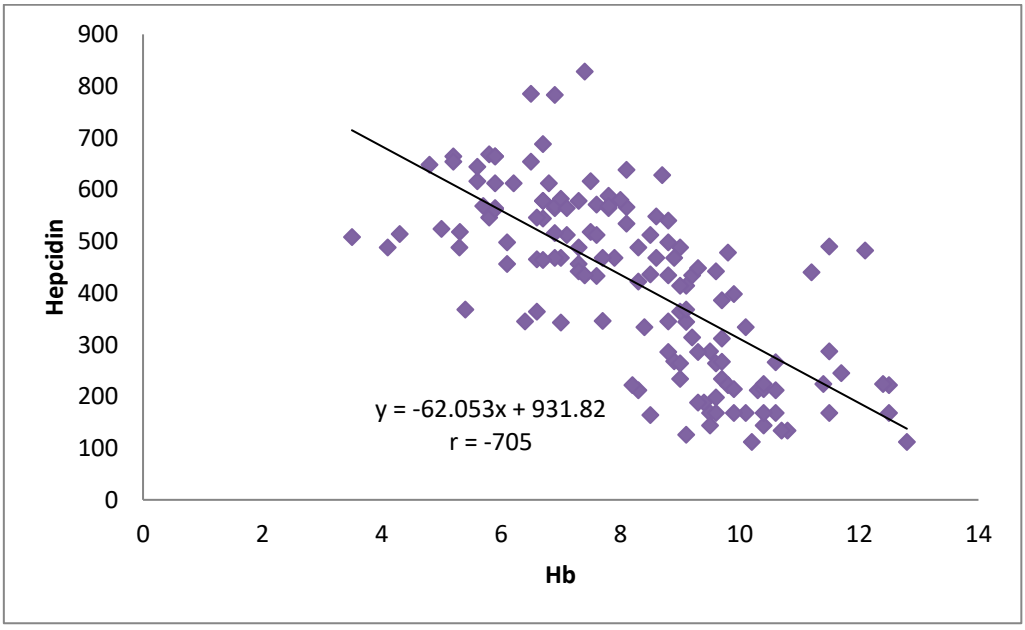
There was a statistically significant mild positive correlation between phosphorous levels and Hb in stage V of CKD

Figure 18: Scatter plot comparison of RET HE and Hemoglobin



There was statistically significant correlation between RET He and Hb in advanced stages of CKD which is associated with functional Iron deficiency. (P=0.05)

Figure 19: Scatter plot comparison of Hemoglobin with Hepcidin



There was a statistically significant moderate negative correlation between hepcidin levels and Hb in stage IIIb of CKD. There was a statistically significant mild negative correlation between hepcidin levels and Hb in stage IV of CKD. There was a statistically significant mild negative correlation between hepcidin levels and Hb in stage V of CKD.

Table 11: Comparison of various parameters in correlation with Hepcidin

Pearson correlation with Hepcidin						
Variables	**CKD**	**Stage II**	**Stage IIIA**	**Stage IIIB**	**Stage IV**	**Stage V**
PTH pg/ml	r value	-.717	.111	-.078	.224	.073
	p value	.070	.717	.758	.160	.578
	N	7	13	18	41	60
ESR	r value	.244	.565*	.149	.176	.217
	p value	.598	.044	.555	.270	.096
	N	7	13	18	41	60
CRP	r value	.081	.181	.190	.268	.253
	p value	.863	.555	.450	.095	.051
	N	7	13	18	40	60
FERRITIN	r value	.702	.291	.789*	.771*	.557*
	p value	.079	.334	.000	.000	.000
	N	7	13	18	41	60
RET HE	r value	-.728	-.427	-.365	-.536*	-.115
	p value	.063	.146	.137	.000	.380
	N	7	13	18	41	60

*- statistically significant by Pearson's correlation coefficient

Figure 20: Scatter plot comparison of PTH and Hepcidin

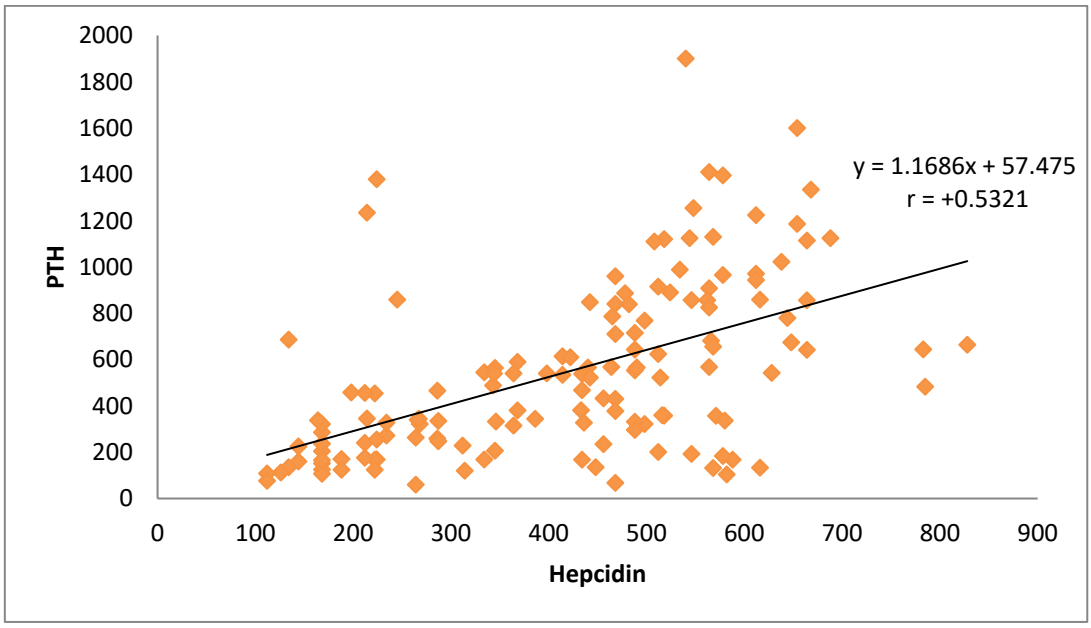
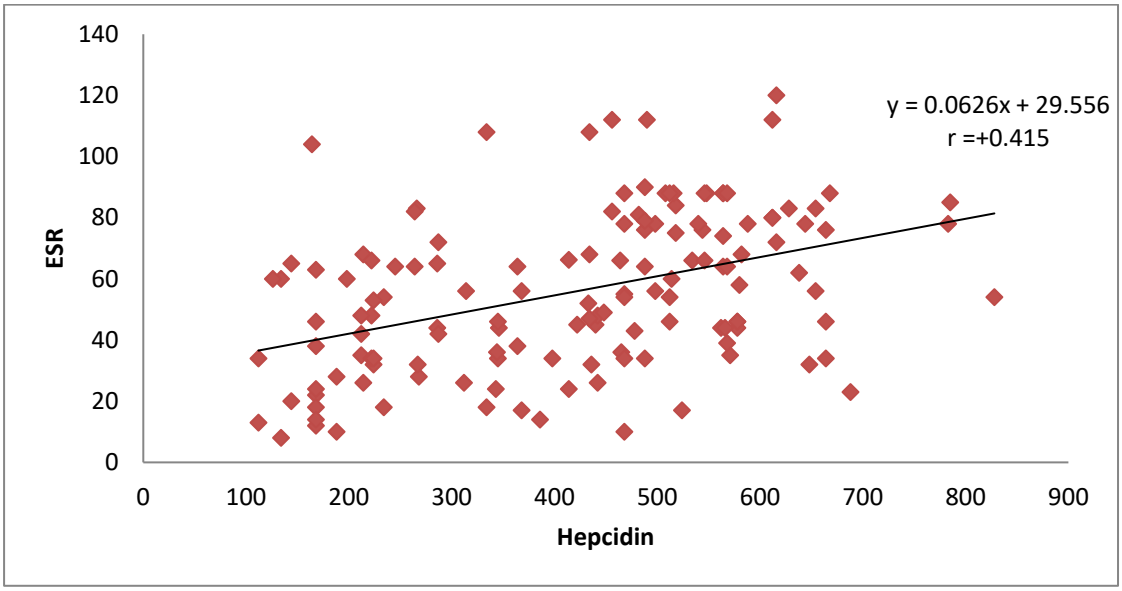
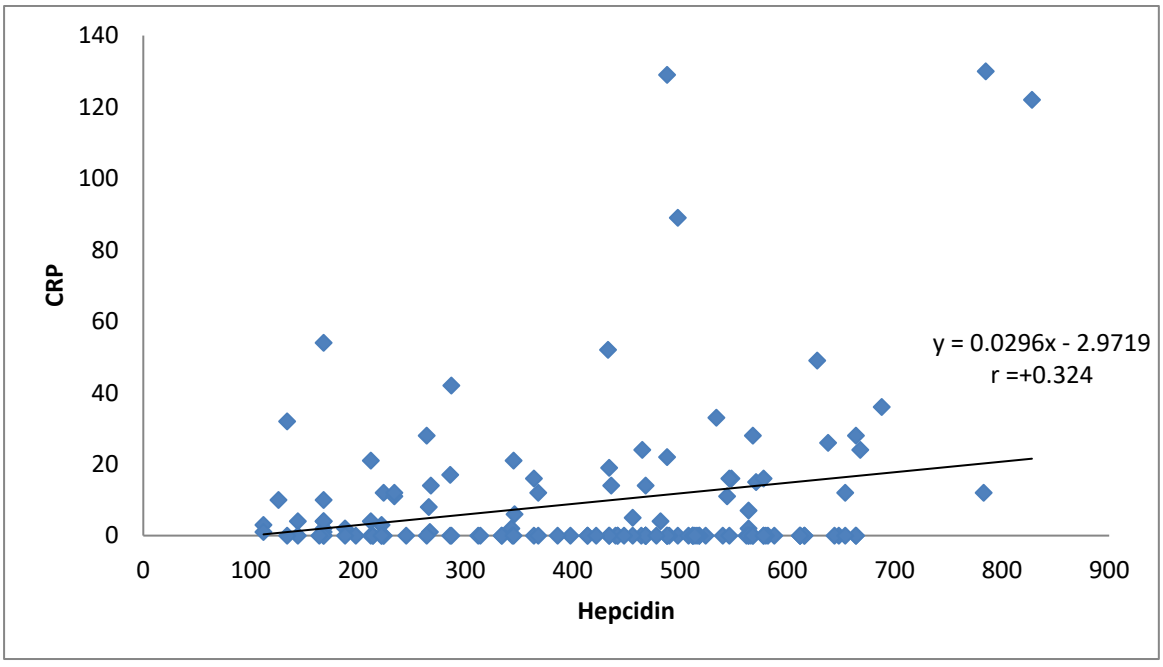


Figure 21: Scatter plot comparison of ESR and Hepcidin



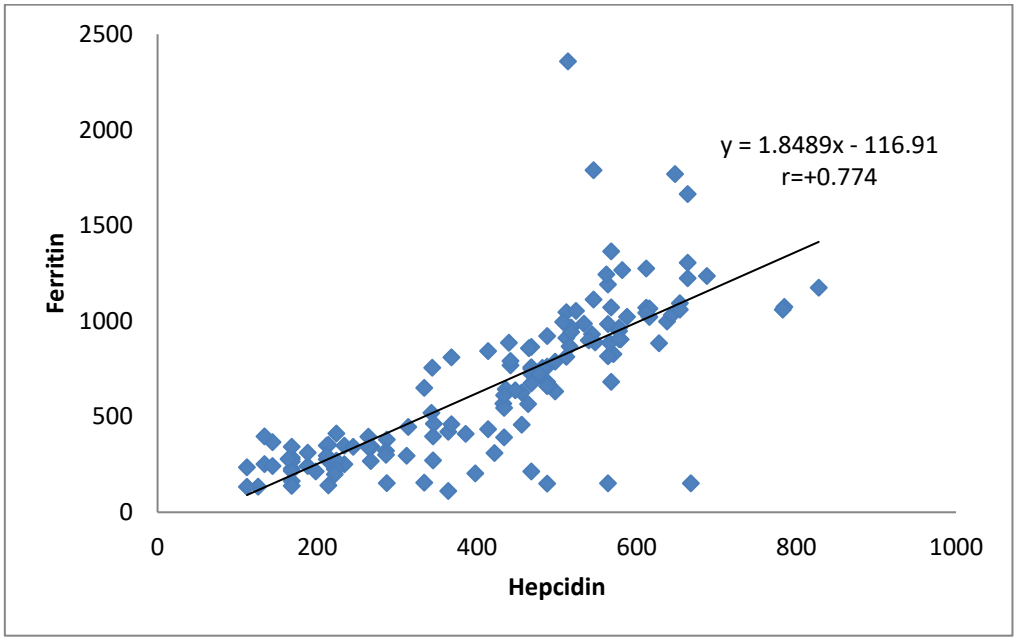
There was a statistically significant moderate positive correlation between ESR and Hepcidin levels in stage IIIA of CKD.

Figure 22: Scatter plot comparison of CRP and Hepcidin



There was no statistically significant correlation between CRP and Hepcidin in various stages of CKD.

Figure 23: Scatter plot comparison of Ferritin and Hepcidin



There was a statistically significant strong positive correlation between ferritin and Hepcidin levels in stage IIIb of CKD. There was a statistically significant strong positive correlation between ferritin and Hepcidin levels in stage IV of CKD. There was a statistically significant moderate positive correlation between ferritin and Hepcidin levels in stage V of CKD.

Figure 24: Scatter plot comparison of RET HE and Hepcidin

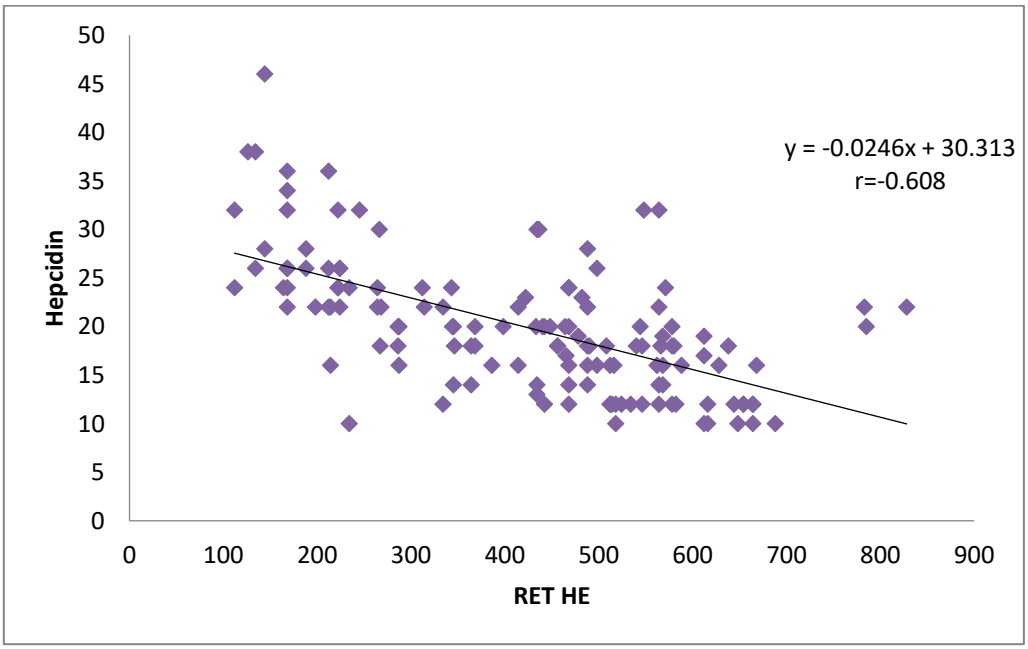


Table 12: Comparison of Ret-He with Iron parameters

Pearson correlation with RET HE						
Variables	**CKD**	**Stage II**	**Stage IIIA**	**Stage IIIB**	**Stage IV**	**Stage V**
IRON	r value	-.612	-.156	.527*	-.195	.071
	p value	.144	.611	**.025**	.221	.589
	N	7	13	18	41	60
TSAT %	r value	-.689	-.043	.336	-.294	-.216
	p value	.087	.889	.173	**.062**	.097
	N	7	13	18	41	60
TIBC	r value	-.249	-.237	.387	.240	.336*
	p value	.590	.437	.113	.131	**.009**
	N	7	13	18	41	60

There is statistically significant negative correlation between Ret-He levels and Serum iron values in Stage III CKD. Transferrin saturation also has statistically significant negative correlation between Ret-He levels

Figure 25: Scatter plot comparison of Iron and Ret He

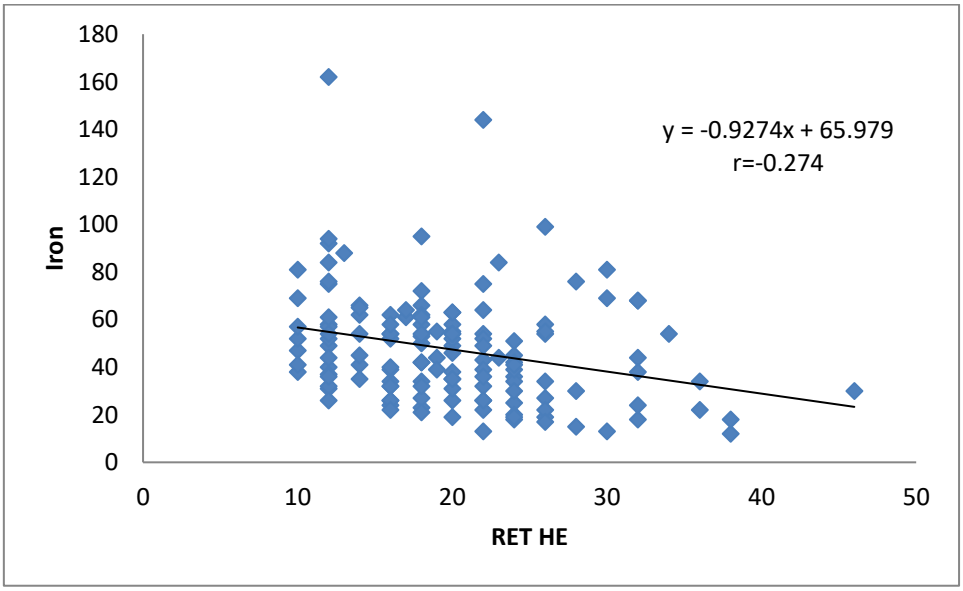
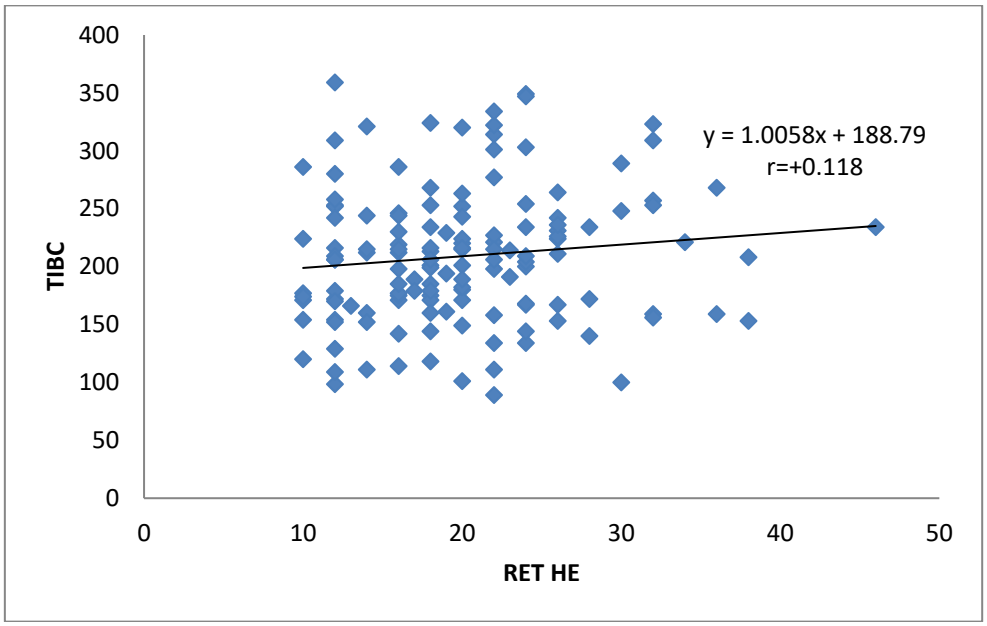


Figure 26: Scatter plot comparison of TIBC and Ret HE



E *Figure 27: Scatter plot comparison of RET HE and TSAT*

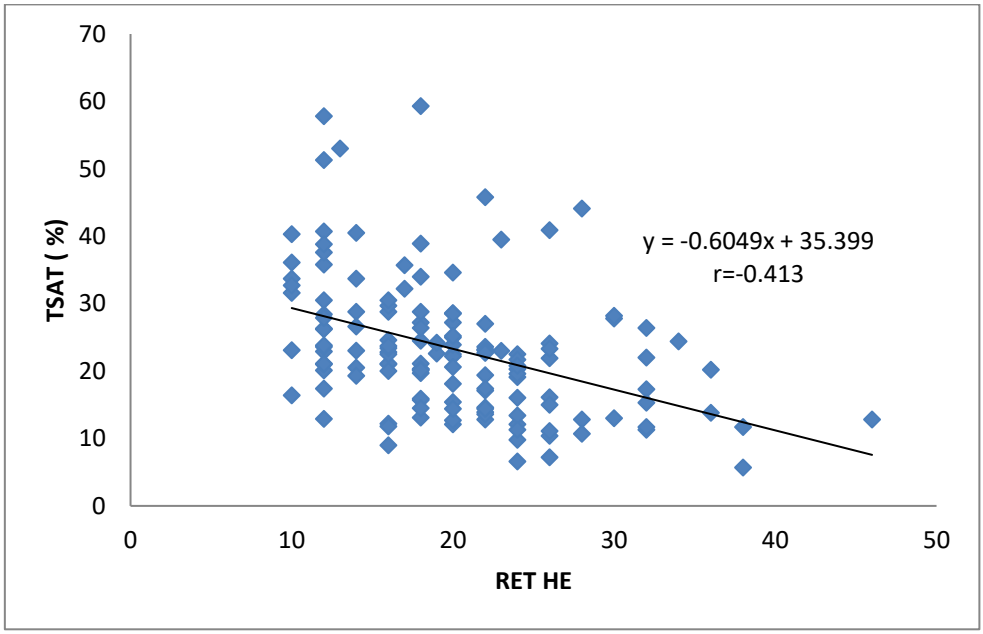


Table 13: Comparison of Absolute and Functional Iron deficiency anemia

Variable (Mean ± SD)	Absolute Iron deficiency (n=53)	Functional Iron deficiency (n=86)	Independent t test value	P value
Age	44.58 ± 17.99	43.10 ± 16.34	0.499	0.619
Hemoglobin	9.74 ± 1.37	7.36 ± 1.62	8.87	**< 0.001***
creatinine	2.92 ± 1.81	7.01 ± 3.42	8.004	**< 0.001***
CrCL (ml/min/m2)	35.56 ± 16.32	14.47 ± 8.81	9.878	**< 0.001***
ESR	46.88 ± 22.80	61.31 ± 25.78	3.346	**0.001***
Hepcidin	259.19 ± 108.68	517.99 ± 117.48	12.974	**< 0.001***
RET HE	24.45 ± 6.79	17.28 ± 5.33	6.928	**< 0.001***
Iron	31.49 ± 12.96	57.23 ± 22.70	7.526	**< 0.001***
TIBC	211.52 ± 62.89	207.31 ± 55.84	0.411	0.682
TSAT%	15.16 ± 4.61	28.30 ± 9.18	9.680	**< 0.001***
Ferritin	326.28 ± 167.72	863.02 ± 372.94	9.869	**< 0.001***
CRP	40.83 ± 22.42	57.48 ± 27.15	2.096	**0.038***
PTH	344.46 ± 176.54	672.58 ± 271.04	5.574	**< 0.001***

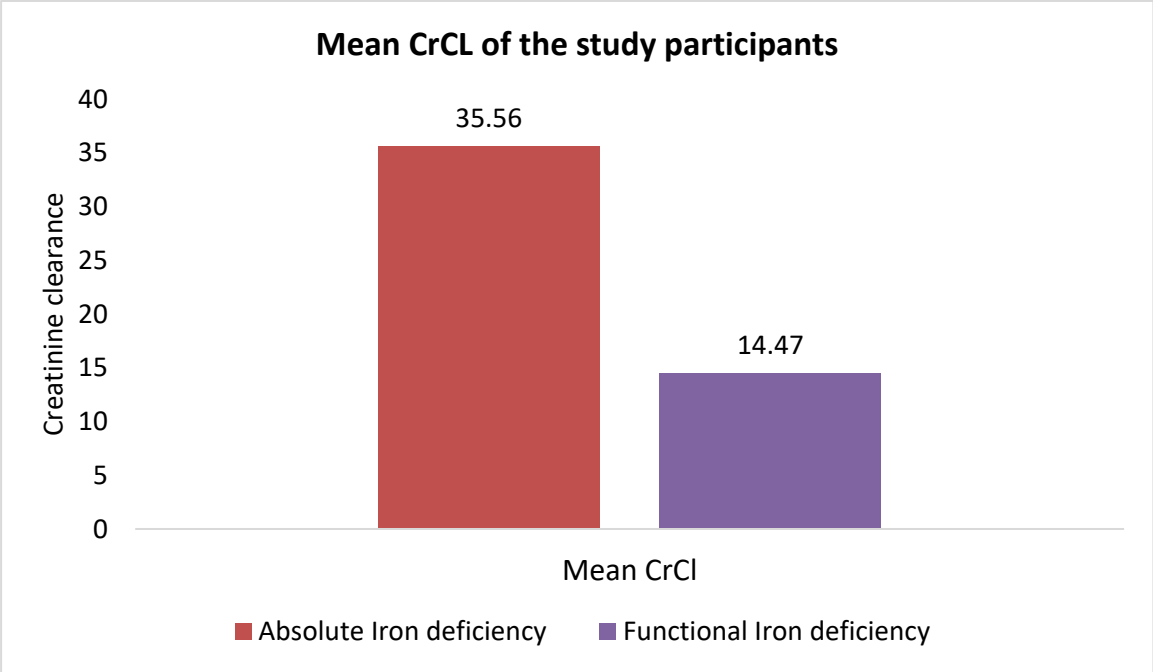
The mean hemoglobin of study participants in participants with absolute iron deficiency was 9.74 ± 1.37 and in participants with functional iron deficiency was 7.36 ± 1.62. This difference was statistically significant by independent sample t test. ($p < 0.05$).

The mean serum creatinine of study participants in participants with absolute iron deficiency was 2.92 ± 1.81 and in participants with functional iron deficiency was 7.01 ± 3.42. This difference was statistically significant by independent sample t test. ($p < 0.05$).

The mean creatinine clearance of study participants in participants with absolute iron deficiency was 35.56 ± 16.32 and in participants with functional iron deficiency was

14.47 ± 8.81. This difference was statistically significant by independent sample t test. ($p < 0.05$).

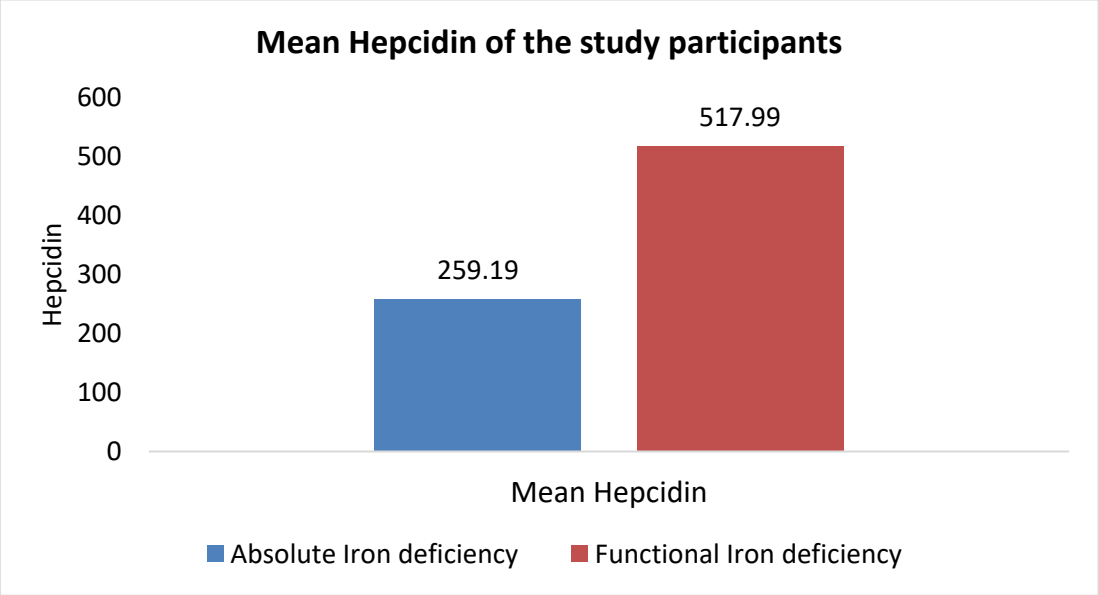
Figure 28: Mean Creatinine clearance in Absolute Iron deficiency and Functional Iron deficiency anemia



The mean ESR of study participants in participants with absolute iron deficiency was 46.88 ± 22.80 and in participants with functional iron deficiency was 61.31 ± 25.78. This difference was statistically significant by independent sample t test. ($p < 0.05$).

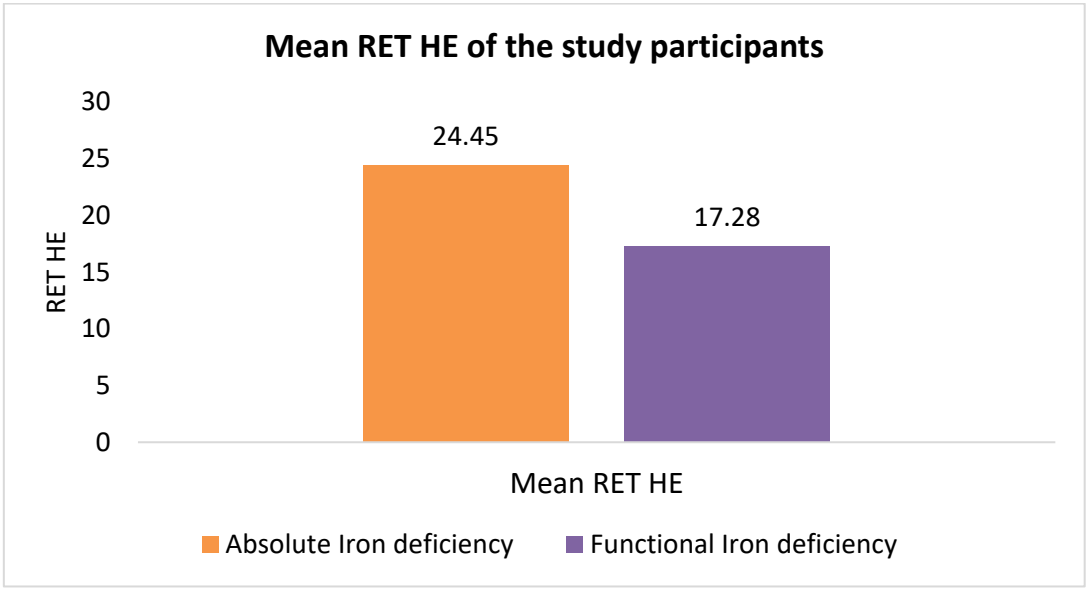
The mean hepcidin levels of study participants in participants with absolute iron deficiency was 259.19 ± 108.68 and in participants with functional iron deficiency was 517.99 ± 117.48. This difference was statistically significant by independent sample t test. ($p < 0.05$).

Figure 29: Mean Hepcidin in Absolute Iron deficiency and Functional Iron deficiency anemia



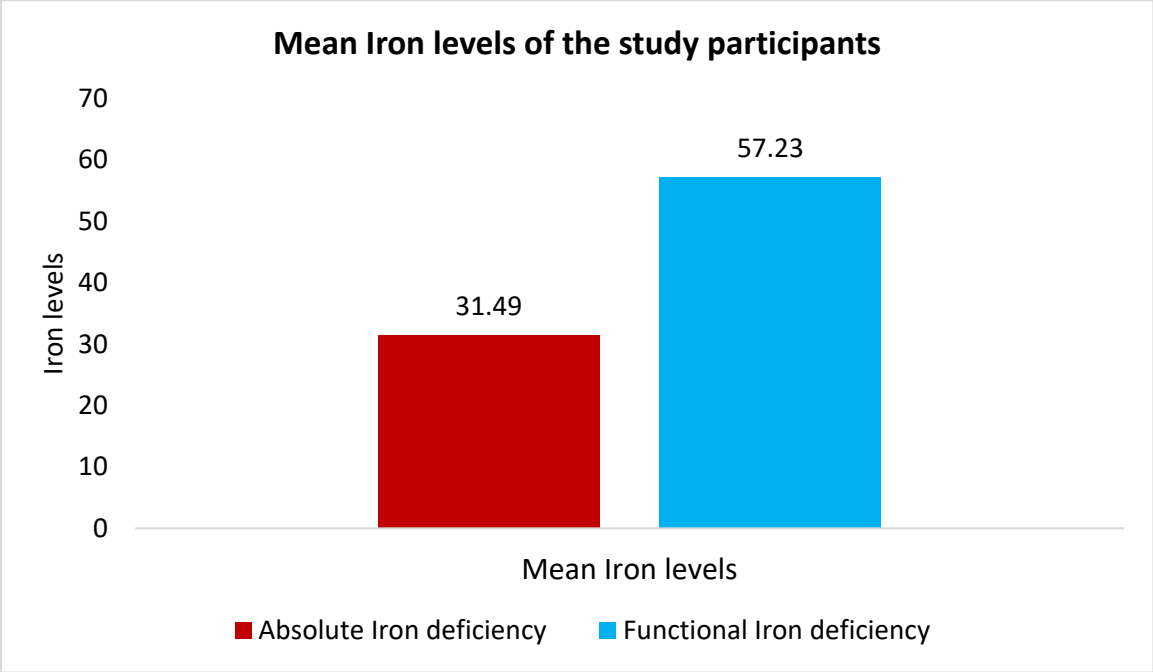
The mean RET HE of study participants in participants with absolute iron deficiency was 24.45 ± 6.79 and in participants with functional iron deficiency was 17.28 ± 5.33. This difference was statistically significant by independent sample t test. ($p < 0.05$).

Figure 30: Mean RET HE in Absolute and Functional Iron deficiency anemia



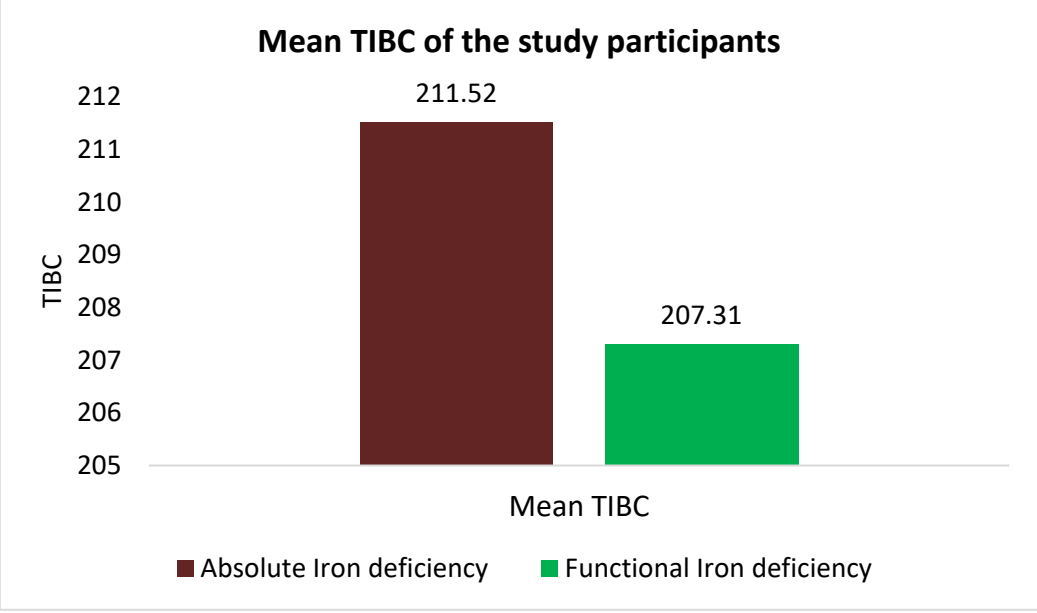
The mean iron levels of study participants in participants with absolute iron deficiency was 31.49 ± 12.96 and in participants with functional iron deficiency was 57.23 ± 22.70. This difference was statistically significant by independent sample t test. ($p < 0.05$).

Figure 31: Mean Iron levels in Absolute and Functional Iron deficiency anemia



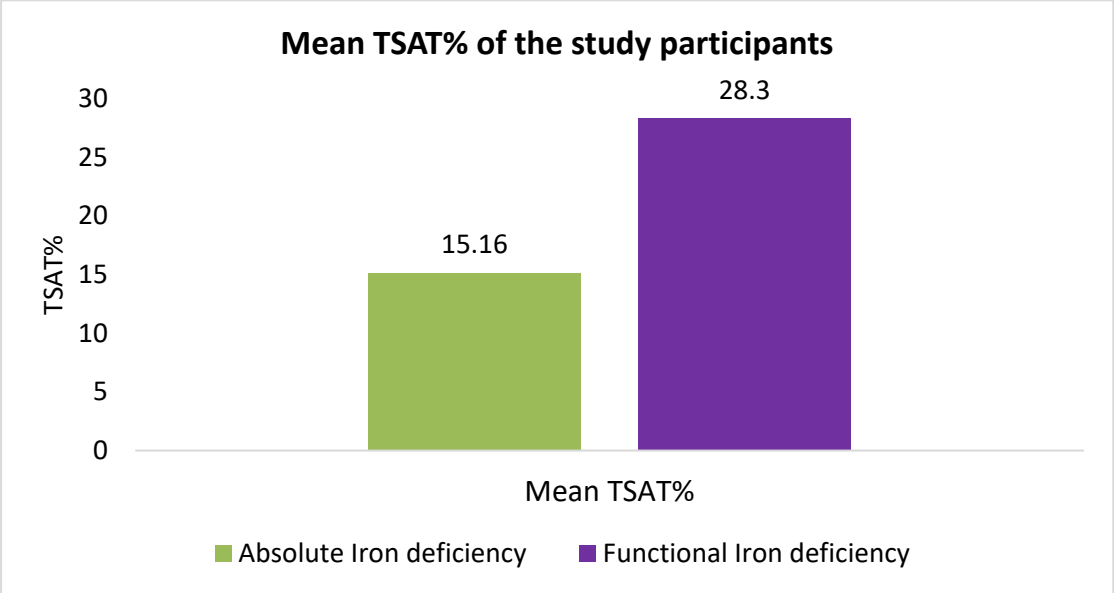
The mean TIBC of study participants in participants with absolute iron deficiency was 211.52 ± 62.89 and in participants with functional iron deficiency was 207.31 ± 55.84. This difference was not statistically significant by independent sample t test.

Figure 32: Mean TIBC in Absolute and Functional Iron deficiency anemia



The mean TSAT% of study participants in participants with absolute iron deficiency was 15.16 ± 4.61 and in participants with functional iron deficiency was 28.30 ± 9.18. This difference was statistically significant by independent sample t test. ($p < 0.05$).

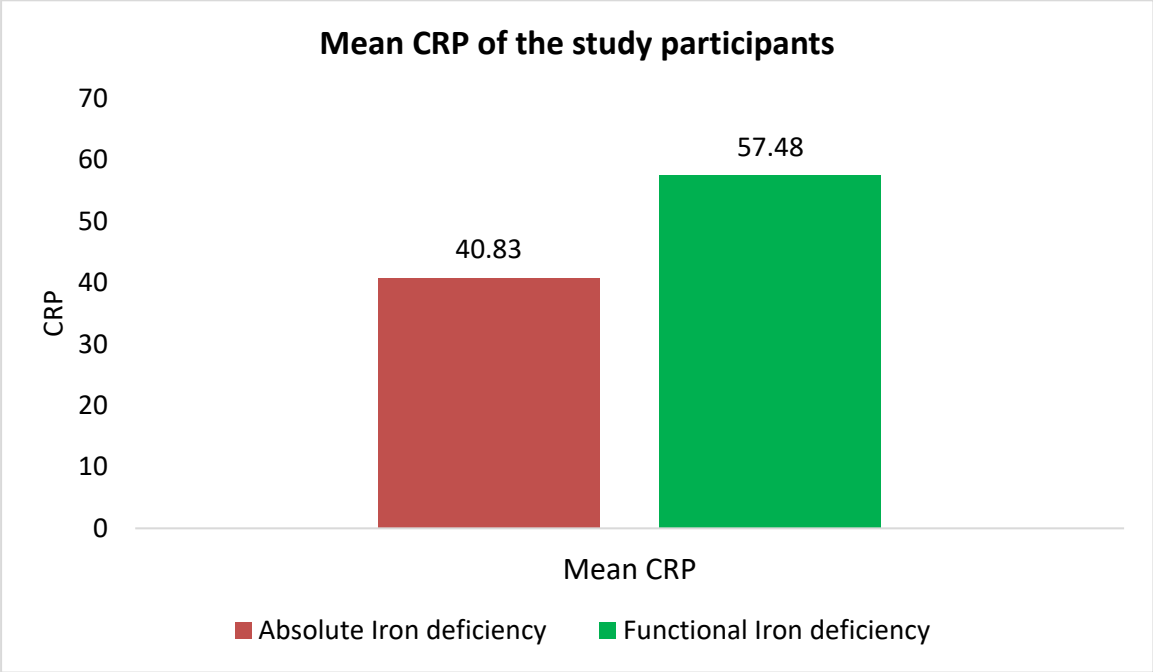
Figure 33: Mean TSAT in Absolute and Functional Iron deficiency anemia



The mean ferritin levels of study participants in participants with absolute iron deficiency were 326.28 ± 167.72 and in participants with functional iron deficiency was 863.02 ± 372.94. This difference was statistically significant by independent sample t test. ($p < 0.05$).

The mean CRP levels of study participants in participants with absolute iron deficiency were 40.83 ± 22.42 and in participants with functional iron deficiency was 57.48 ± 27.15. This difference was statistically significant by independent sample t test. ($p < 0.05$).

Figure 34: Mean CRP in Absolute and Functional Iron deficiency anemia



The mean PTH levels of study participants in participants with absolute iron deficiency were 344.46 ± 176.54 and in participants with functional iron deficiency was 672.58 ± 271.04. This difference was statistically significant by independent sample t test. ($p < 0.05$).

DISCUSSION

This is an observational cross-sectional study which was done to establish the correlation of Hepcidin in different stages of CKD with various parameters that contribute to anemia in CKD.

Due to the combination of reduced iron absorption and increased iron losses, iron deficiency is common among CKD patients who are both non-Dialysis and dialysis dependent. The following two forms of iron deficiency are recognized: Absolute (true) iron deficiency and Functional iron deficiency. Absolute iron deficiency is defined by severely reduced or absent iron stores in bone marrow, liver, and spleen. Functional iron deficiency is defined by normal or increased total body iron stores which are unavailable for incorporation into erythroid precursors for erythropoiesis [37]. Functional iron deficiency is mainly due to increased levels of hepcidin which reduce the ability to recruit iron stores from reticuloendothelial cells and hepatocytes for erythropoiesis.

In a meta-analysis of RCT conducted in 2018[38], with 24 trials included, 13 of which were in the CKD stage 3–5 ND population. The 13 CKD stage 3–5 ND trials were heterogeneous about inclusion criteria. The Hb threshold criteria ranged from <8 to <12 g/dL, the TSAT threshold criteria was either <20 or 25 or 30% and the ferritin threshold ranged from <100 to <600 ng/mL. Subsequently, trials of both functional and absolute iron deficiency were included. Accordingly, the baseline hematologic parameters varied: Hb concentrations varied from 5.8 ± 0.6 to 11.9 ± 0.7 g/ dL, baseline ferritin levels varied from 57.3 ± 48.6 to 345 ± 273 ng/mL, and baseline TSAT varied from 15.4 ± 5.5 to 63.6 ± 11.1% [39]. In our study, the threshold of Hb ranged from <5 to <12 g/dl , the mean iron values in stage IIIB,IV and V were 28.85 mg/dl,48.10 mg/dl and 57.52 mg/dl respectively ,TSAT threshold criteria was < 20 % and ferritin threshold ranged from 200 to 2000 ng/ml with Stage V CKD patients having a mean of 913.92 ng/ml with a p value of <0.001 which made it statistically significant in comparison with Iron, TIBC and ferritin levels in advanced CKD .

Awan et al [40] in 2021 showed that in a study of 993 patients cared for at the Department of Veterans Affairs from 1 January 2005 to 31 December 2015, 463 patients with CKD, 20.6% had anemia. Among those with anemia, 23.6% of patients

had both TSAT and ferritin level measured, of whom 30% had absolute IDA and 19% had functional IDA. Absolute IDA in CKD was not associated with an increased risk of mortality or dialysis but was associated with a higher risk of 1-year {risk ratio [RR] 1.20 [95% confidence interval (CI) 1.12–1.28]} and 2-year cardiovascular hospitalization [RR 1.11 (95% CI 1.05–1.17)]. CKD patients with functional IDA had a higher risk of mortality [hazard ratio (HR) 1.11 (95% CI 1.07–1.14)] along with a higher risk of 1- year [RR 1.21 (95% CI 1.1–1.30)] and 2-year cardiovascular hospitalization [RR 1.13 (95% CI 1.07–1.21)]. Ferritin >500 ng/ mL (treated as a separate category) was only associated with an increased risk of mortality [HR 1.38 (95% CI 1.26–1.51)] In our study, we found out that Absolute iron deficiency was found in 30.8 % in CKD stage III A, 44.4 % in stage III B , 26 .8 % in stage IV and 21.7 % in stage V CKD patients , while Functional iron deficiency was found in 69.2 % in stage III A, 55.6 % in stage III B , 73.2 % in stage IV and 78.3 % in stage V . The study by Awan et al showed that Functional IDA was associated with an increased risk of mortality and cardiovascular hospitalization, but absolute IDA was associated only with a higher risk of hospitalization. In our study, we found that the Prevalence of Functional Iron deficiency was much higher in stage V CKD which can be attributed to higher levels of Hepcidin in stage V (mean of 543.47 ng/ml).

The gold standard in assessing iron deficiency is bone marrow staining using Prussian Blue, but this examination is invasive, therefore hematology and biochemical parameters are commonly used. Hematology parameters can only detect advanced stages of iron deficiency, while biochemical parameters such as serum iron, transferrin, ferritin are affected by inflammation. The development of flow cytometry in the latest automated hematology analyzers can estimate the hemoglobin content of reticulocytes (Reticulocytes Hemoglobin Equivalent/ Ret- He). Ret-He can give information on how much iron is available for the erythropoiesis in the bone marrow and can detect iron deficiency in earlier stages. Reticulocytes have a more rapid turnover in circulation compared to mature cells, suggesting that Ret-He is more sensitive in assessing erythropoietic activity [41,42] Ret-He test is easier to perform and relatively cheaper than other iron profile tests.

In a study conducted by Grover et al [43], of 66 patients 15 patients had IDA, 19 had EPO deficiency and 35 had FID, Ret-he was most sensitive (85.7%) for diagnosing IDA

at a cut off of 27.2pg. Serum ferritin was most specific (87%) for diagnosing IDA at a cut off value of 100mcg/dl These results suggest low sensitivity and specificity of serum iron and TSAT in diagnosing IDA and FID and low sensitivity of serum ferritin in diagnosis iron deficiency anemia. In our study, Mean Ret-he levels in stage III, IV, V of CKD were 24,20.02 and 16.35 respectively and showed a statistically significant association with Hemoglobin (positive co relation with r value of 0.253 and P value of 0.053) in advanced renal failure.

From the Pearson TSAT and Ret-he correlation table obtained a significance value of <0.001, it can be concluded that there is a relationship between Ret-he and TSAT levels with a significance value of <0.05, According to the Pearson correlation number of 0.618 which is in the range of 0.61 to 0.80, it can be concluded that there is a strong correlation between the two variables. This is in line with the study conducted by Miwa et al. (2010) which showed a good correlation between Ret-he and TSAT ($r=0.543$, $p<0.01$) [44]. Ret HE is the most sensitive marker for diagnosing both iron deficiency and functional iron deficiency is patients of CKD, As Ret-he cannot differentiate between IDA and FID so it should be used along with serum ferritin to diagnose the cause of anemia in CKD patients, in this study, we report the usefulness of the Ret-HE parameter as an index of iron status in HD patients. This finding agrees with another study, in which RET-He showed excellent diagnostic performance and was generally superior for the diagnosis of iron deficiency anemia among HD patients when compared with traditional parameters, with a cut-off rate of 26.0 picograms.

Functional iron deficiency secondary to inflammation and increased serum hepcidin lead to erythropoietin-resistant anemia in children with chronic kidney disease. Vitamin D deficiency, parathyroid hormone, and phosphate can also participate in chronic inflammation and anemia. In patients with CKD, vitamin D deficiency, and high intact parathyroid hormone (intact PTH) and phosphate levels may contribute to chronic inflammation and increase of hepcidin, which consequently lead to ESA-resistance anemia due to the decreased bone marrow production of red blood cells (RBCs) [37]. Fibroblast growth factor 23 (FGF23) is a phosphate-regulating hormone primarily secreted by osteocytes and osteoblasts. It is increased in CKD secondary to high phosphate levels and leads to increase in intact PTH indirectly by decreasing 1,25-dihydroxy vitamin D [1,25(OH)2 D] synthesis. It decreases the phosphate reabsorption

in renal proximal tubules and inhibits absorption of phosphate in the intestine by reducing active vitamin D synthesis.

Recent studies suggested that vitamin D deficiency might play an important role increasing hepcidin levels and correlated inversely with the ESA-resistance anemia in patients with CKD (38). Vitamin D has been shown to decrease circulating hepcidin levels in healthy volunteers and patients undergoing hemodialysis. Increased intact PTH has a similar effect on hepcidin. Treatment with vitamin D and calcitriol was reported to suppress inflammation and improve the effectiveness of EPO therapy in healthy adults and patients with CKD. In our study, the decrease in vitamin D levels in advancing stages of CKD was statistically significant ($p < 0.05$) and the increase in PTH levels in advancing stages of CKD was statistically significant by ANOVA test ($p < 0.05$),}. There was a statistically significant mild positive correlation between phosphorous levels and Hb in stage V of CKD ($p = 0.046$) but there was no statistically significant correlation between PTH and Hepcidin in various stages of CKD {p value = 0.889, r = +0.5321}.

The relationship between hepcidin and inflammatory mediators has not been consistently demonstrated in patients with CKD. Malyszko and Ganz et al. (45) demonstrated a correlation between hepcidin and CRP. By contrast, many studies have not reported this correlation (46). According to numerous studies, IL-6 is apparently a key inducer of hepcidin synthesis during inflammation, whereas IL-1 or tumor necrosis factor alpha did not affect hepcidin synthesis. On the other hand, Camaschella et al. (47) reported that inflammatory cytokines, namely IL-6, Interleukin- 1, TNF-and gamma-interferon were involved in activation of hepcidin production and found that IL-6 was the most important cytokine in the pathogenesis of the anemia of chronic disease. Similarly, we found increased levels of inflammatory molecules of ESR, CRP, Ferritin in patients with CKD. Ferritin and ESR correlated with hepcidin levels, whereas no correlation was observed between CRP, and hepcidin. None of our patients had additional acute or chronic inflammatory conditions other than CKD in the study group. According to our results, we suggest that inflammation of CKD may also show a marked effect on serum hepcidin levels if there are concomitant acute or chronic inflammatory conditions. The etiology of CKD may also play a role in inflammatory situation. In few of our patients, the underlying cause of CKD was CAKUT. Therefore, chronic inflammation may be less important in

patients with CAKUT in the early stages of CKD. We could not perform statistical analysis according to the etiology of CKD because we had a small number of patients.

Although our study has potential limitations due to its cross-sectional design and small sample size, to our knowledge, this is the first study to evaluate the relation between bone-mineral metabolism, anemia, inflammation, and hepcidin, Ret-he in CKD patients across different stages. our data suggest that serum hepcidin levels are elevated significantly in CKD patients that had a strong positive correlation with ESR, Ferritin, (p=<0.001) and equally strong negative correlation with hemoglobin(p=0.003) and, thus contributing to functional iron deficiency anemia. The increase of serum hepcidin levels may be inhibited by effective treatment of anemia and secondary hyperparathyroidism with phosphate binders and the active form of vitamin D, which decrease serum intact PTH, FGF-23, and phosphorus levels, and control inflammation to some extent.

Lack of standardized definitions of absolute and functional iron deficiency in those with non-dialysis-dependent CKD limits comparability with prior studies. For instance, a National Health and Nutrition Examination Survey (NHANES) analysis (1988–94) (n¼15 837) noted that among those with a creatinine clearance of 20–30mL/min, 46% of women and 19% of men had a TSAT <20%, while 47% of women and 44% of men had serum ferritin <100 ng/mL [48]. However, patients were not categorized into absolute or functional IDA. In our study, we found that the mean hemoglobin of study participants in participants with absolute iron deficiency was 9.74 ± 1.37 and in participants with functional iron deficiency was 7.36 ± 1.62(P<0.01) which is like a study by Sanjay Vikrant et al [49], which showed a mean hemoglobin was 9.2 ± 2.2 g/dL. There was a progressive fall in hemoglobin with increasing severity of CKD and in CKD Stage 3, 4, and 5 the mean hemoglobin was 10 ± 2.2, 9.4 ± 2.1, and 8.4 ± 1.9 g/dL, respectively. One hundred and sixty-two (27.7%) patients had serum ferritin <100 ng/mL (absolute iron deficiency); 334 (57.2%) patients had serum ferritin 100-500 ng/mL, but in 175 (52.4%) of them, TSAT was <20%; 88 (15.1%) patients had serum ferritin >500 ng/mL (58 (65.6%) were C-reactive protein (CRP) + and 55 (62.5%) had TSAT <20%). Overall, 392 (67.1%) patients had functional or absolute iron deficiency. The mean hepcidin levels of study participants in participants with absolute iron deficiency was 259.19 ± 108.68 and in participants with functional iron deficiency was

517.99 ± 117.48. This difference was statistically significant by independent sample t test. ($p < 0.05$) The mean iron levels of study participants in participants with absolute iron deficiency was 31.49 ± 12.96 and in participants with functional iron deficiency was 57.23 ± 22.70. The mean TIBC of study participants in participants with absolute iron deficiency was 211.52 ± 62.89 and in participants with functional iron deficiency was 207.31 ± 55.84. This difference was not statistically significant by independent sample t test. The mean TSAT% of study participants in participants with absolute iron deficiency was 15.16 ± 4.61(73.67%) and in participants with functional iron deficiency was 28.30 ± 9.18(. This difference was statistically significant by independent sample t test. ($p < 0.05$) The mean ferritin levels of study participants in participants with absolute iron deficiency was 326.28 ± 167.72 and in participants with functional iron deficiency was 863.02 ± 372.94 which was statistically significant by independent sample t test. ($p < 0.05$) The mean CRP levels of study participants in participants with absolute iron deficiency was 40.83 ± 22.42 and in participants with functional iron deficiency was 57.48 ± 27.15. This difference was statistically significant by independent sample t test. ($p < 0.05$).

LIMITATION OF THE STUDY

This present study has a few limitations, Firstly, the sample size was not very large and it is a single centered study. During the chronic inflammatory state, immune cells such as macrophages and T-lymphocytes produce more proinflammatory cytokines, especially IL-6, which in turn is responsible for the production of hepcidin. Several previous studies had determined a highly significant correlation between hepcidin and IL-6 levels in CKD patients and the probable role of interleukins and inflammatory cytokines, particularly interleukin-6 (IL-6) in pathogenesis of Anemia in CKD [50,51] In our study, Interleukin -6 levels were not estimated, thus we could not establish relation of Hepcidin with IL-6[52]. Anemia in advanced CKD is usually multifactorial and dependent on the underlying etiology as well as the nutritional status of the patient. In our study, we have not included the etiology of chronic kidney disease as well as nutritional parameters in chronic kidney disease. Hepcidin assays in our study were done by using c-ELISA (competitive ELISA) which is the method of choice for the large-scale quantification of serum hepcidin concentrations, because of its low limit of detection, low cost, and high-throughput, but lacks specificity. Assay for bioactive hepcidin-25 by WCX-TOF-MS can be regarded as a valuable special-purpose assay for disorders with variable concentrations of hepcidin isoforms, such as chronic kidney disease weak cation exchange time-of-flight mass spectrometry (WCX-TOF MS). Measuring the Ret-he can predict the iron status in respond intravenous (IV) iron supplementation in CKD patients, this study was a cross sectional study and hence patients were not followed up after correction after their anemia, hence we could not stratify Ret-he and Hepcidin levels post erythropoietin. Hence, we could not compare Ret-he and hepcidin levels post therapy which might give us a clue regarding the statistical significance on follow up

CONCLUSION

- Iron Deficiency Anaemia is a common complication in chronic kidney disease (CKD), and is associated with a reduced quality of life, and an increased morbidity and mortality. The mechanisms involved in anaemia associated to CKD are diverse and complex.

- These include a decrease in endogenous erythropoietin (EPO) production, absolute and/or functional iron deficiency, and inflammation with increased hepcidin levels.

- Serum Hepcidin levels significantly correlates with Functional Iron deficiency in advanced chronic kidney disease and can be used as a marker of Functional iron deficiency anaemia. As CKD progresses, hepcidin level increases regardless of the inflammatory state.

- Having established the central role of hepcidin in iron homeostasis, it is possible to consider its use as a novel treatment for anemia in CKD patients.

- Hepcidin antagonists could be used in patients with diseases that cause hepcidin excess and occur with a framework of IDA or systemic IDA.

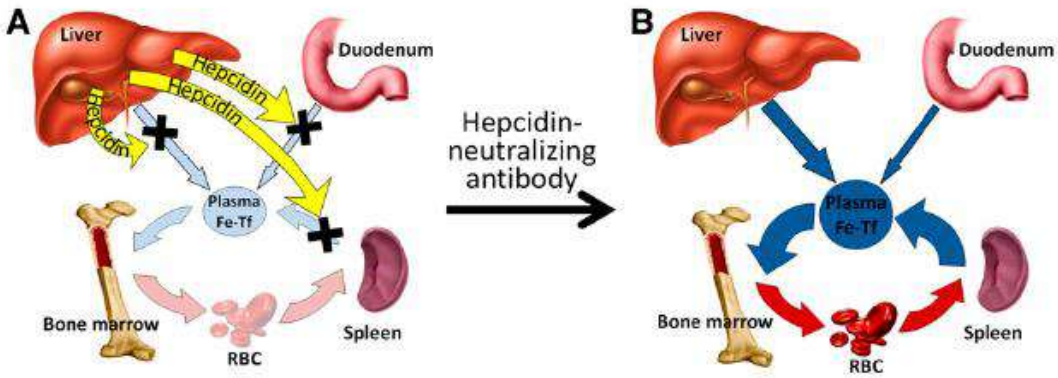
- Hydroxyproline inhibitors may be effective for inducing hepcidin inhibition; such inhibition could be caused not only by erythropoiesis stimulation but also by the ability of hydroxyproline inhibitors to interfere with the transcriptional complex, hypoxia-inducible factor

- Ret-he is a relevant marker of iron status among patients with early stages of CKD and correlates well with anemia indices which could help identify more patients with iron deficiency.

- Ret-He had a significant correlation with Iron and Transferrin saturation. The correlation strength of Ret-He, and iron as well as Ret-He and transferrin saturation, were moderate while Ret-he and TIBC had a weak correlation, these tests suggest that Ret-He may be used to assess iron status of patients with CKD and correlates well with anemia indices which could provide a more rapid and precise diagnosis.

What the Future holds?

- ➢ Research studies were conducted to develop therapeutic agents inhibiting hepcidin to enhance iron utilization from iron storage. Anti-hepcidin antibody was used in mice to validate the efficacy of this agent to inhibit hepcidin [53].
- ➢ In the first human clinical trial, Van Eijk et al used an antihepcidin peptide, Spiegelmer lexaptepid, and detected the effectiveness of lexaptepid in preventing systemic inflammations and thus suggested lexaptepid to be a promising therapeutic agent for functional Iron deficiency anemia [54]



Picture 5: Anti-hepcidin therapy in iron-restricted anaemias

- ➢ The efficacy of hepcidin antagonists in treating iron-restricted anemia in humans remains to be tested in clinical trials, several of which are in progress. Additional therapeutic options may soon become available for patients with anemias associated with kidney disease, cancer, and other inflammatory conditions
- ➢ Antibodies that block ferroportin binding to hepcidin without affecting its functionality have been described (Leung et al.,55). They have been engineered and are now in a Phase I trial. A high throughput screening approach discovered a thiol modifier compound (fursultiamine) that prevented ferroportin–hepcidin interaction sequestering the Cys326-HS residue (essential for hepcidin binding, Figure 3A) and blocking internalization of ferroportin (Fung and Nemeth,56). It could be an interesting agent to be evaluated in vivo.

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ANNEXURE-1

GOVERNMENT OF INDIA

ALL INDIA INSTITUTE OF MEDICAL SCIENCES,

JODHPUR-342005, INDIA

INFORMED CONSENT FORM

I _____ s/d/w of _____ a resident of _____

hereby declare that I give informed consent to participate in the Thesis study labelled **"STUDY OF THE ASSOSCIATION OF HEPICIDIN IN ABSOLUTE AND FUNCTIONAL IRON DEFICIENCY ANEMIA IN CKD PATIENTS".** Dr. SANTOSH KUMAR V has informed me to my full satisfaction, in the language I understand, about the purpose, nature of study and various investigations to be carried out for the study. I have been informed about the duration of the study and possible complications caused by study.

I give full consent for being enrolled in the above study and I reserve my rights to withdraw from the study whenever I wish without prejudice of my right to undergo further treatment at this hospital and its associated hospitals.

_____ _____

Name of Subject Date Signature of subject

We have witnessed that the patient signed the above form in the presence of his/her free will after fully having understood its contents.

_____ _____

Name of Witness Date Signature of witness

_____ _____

Name of Investigator Date Signature of Investigator

ANNEXURE-2

अखिल भारतीय आयुर्विज्ञान संस्थान

जोधपुर, राजस्थान

सूचित सहमति प्रपत्र

थीसिसकाशीर्षक "**STUDY OF THE CORRELATION OF HEPICIDIN IN ABSOLUTE AND FUNCTIONAL IRON DEFICIENCY ANEMIA IN CKD PATIENTS**" द्वारा डॉ सन्तोष कुमार वी

रोगी / स्वयंसेवी पहचान संख्या.: _____

मैं, _____ पुत्र/पुत्री _____

निवासी _____ मेरीपूर्ण, नि: शुल्क, स्वैच्छिक सहमति देता हु निम्नलिखित अध्ययन का हिस्सा बनने के लिए जिसकी प्रक्रिया और प्रकृति मेरी पूरी संतुष्टि के,किसी भी समय अध्ययन सेबाहर निकलने के मेरे अधिकार से अवगत हूं.मैं समझता हूं कि मेरे और मेरे किसी भी मेडिकल रिकॉर्ड के बारे में एकत्र की गई जानकारी एम्स जोधपुर सेयानियाम प्राधिकरणों सेजिम्मेदार व्यक्ति द्वारा देखी जा सकती है।मैं इन व्यक्तियों के लिए अपने रिकॉर्ड तक पहुंचने की अनुमति देताहूं।

दिनांक: _____ _____

स्थान : _____ हस्ताक्षर/बाएंअंगूठेकीछाप

तारीख :_____ _____

स्थान: _____ हस्ताक्षर

यह प्रमाणित करने के लिए कि उपर्युक्त सहमति मेरी उपस्थिति में प्राप्त की गई है।

साक्षी1

हस्ताक्षर: _____

नाम: _____

स्थान : _____

साक्षी2

हस्ताक्षर: _____

नाम: _____

स्थान : _____

ANNEXURE-3

PATIENT INFORMATION SHEET

Name of the patient: **Patient ID.:**

"STUDY OF THE CORRELATION OF HEPICIDIN IN ABSOLUTE AND FUNCTIONAL IRON DEFICIENCY ANEMIA IN CKD PATIENTS".

1. **Aim of the study**:
   - To compare the various biochemical parameters of iron studies in absolute and functional IDA in CKD patients.
   - Quantitative assessment of serum Hepcidin levels and to study correlation of these parameters with absolute and functional IDA in CKD patients.
2. **Study site:** Out Patient and in-patient services of Department of Nephrology, All India Institute of Medical Sciences, Jodhpur, Rajasthan.
3. **Study procedure:** After detailed history, clinical examination and necessary baseline laboratory investigations, patients will be diagnosed as CKD with IDA. Timely clinical and laboratory monitoring will be done.
4. **Likely benefit:** Study will help to know the correlation of Hepcidin IN CKD patients with IDA.
5. **Confidentiality:** All the data collected from each study participant will be kept highly confidential.
6. **Risk:** Enrolment in above study poses no substantial risk to any of the study participant.
7. **Withdrawal from study**: You are free to decide whether to participate or not in the study or withdraw from the study anytime. If you choose not to participate in the study or withdraw from the study, you will continue to receive the same amount of care and treatment at AIIMS, Jodhpur.

ANNEXURE-4

रोगी सूचना पत्रक

रोगी का नाम: रोगी आईडी :

अध्ययन का उद्देश्य:

1. अध्ययन का उद्देश्य:

Patients सीकेडी रोगियों में निरपेक्ष और कार्यात्मक आईडीए में लोहे के अध्ययन के विभिन्न जैव रासायनिक मापदंडों की तुलना करना। सीरम हेपसीडिन के स्तर का मात्रात्मक मूल्यांकन और सीकेटी रोगियों में निरपेक्ष और कार्यात्मक आईडीए के साथ इन मापदंडों के सहसंबंध का अध्ययन करना।

2. अध्ययन स्थल: अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर, राजस्थान के डिपार्टमेंट ऑफ नेफ्रोलॉजी के रोगी और बाहर की रोगी सेवाएं।

3. अध्ययन प्रक्रिया: विस्तृत इतिहास, नैदानिक परीक्षा और आवश्यक आधारभूत प्रयोगशाला जांच के बाद, रोगियों को आईडीए के साथ सीकेडी के रूप में निदान किया जाएगा। समय पर नैदानिक और प्रयोगशाला निगरानी की जाएगी।

4. संभावित लाभ: अध्ययन आईडीए के साथ सीकेडी रोगियों में हेपसीडिन के सहसंबंध को जानने में मदद करेगा।

5. गोपनीयता: प्रत्येक अध्ययन प्रतिभागी से एकत्र किए गए सभी डेटा को अत्यधिक गोपनीय रखा जाएगा।

6. जोखिम: उपरोक्त अध्ययन में नामांकन अध्ययन के किसी भी प्रतिभागी के लिए कोई जोखिम नहीं है।

7. अध्ययन से पीछे हटना: आप अध्ययन में भाग लेने या न लेने का निर्णय लेने के लिए स्वतंत्र हैं या अध्ययन से कभी भी पीछे हट सकते हैं। यदि आप अध्ययन में भाग नहीं लेते हैं या अध्ययन से पीछे हटते हैं, तो आपको एम्स, जोधपुर में देखभाल और उपचार की समान मात्रा प्राप्त होती रहेगी।

अधिक जानकारी / प्रश्नों के लिए, निम्नलिखित कर्मियों से संपर्क किया जा सकता है: डॉ सन्तोष कुमार वी, नेफ्रोलॉजी विभाग, ऑल इंडिया इंस्टीट्यूट ऑफ मेडिकल साइंसेज, जोधपुर, राजस्थान। पीएच: 9884483982

ANNEXURE 5

PROFORMA

Name: **Study Serial No.:**

Age: **Sex:**

Reg No.:

Address: **Phone No.:**

Diagnosis:

Duration of CKD **eGFR**

LABORATORY (BIOCHEMICAL)

Date	Baseline
HB/TLC/PLT	
ESR/CRP	
Na/K/Cl	
Urea /Cr	
AST/ALT/ALP	
Bilirubin (T/D)	
Ca/phosphorous	
Hepcidin/Reti-he	
PERIPHERAL SMEAR	
Vit D/PTH	
B12	
Iron/TIBC/Ferritin	

ANNEXURE 6

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2021/3496 Date: 12/03/2021

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3331**

Project title: **"Study of the correlation of hepcidin in absolute and functional iron deficiency anemia in CKD patients"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**D.M. Dissertation**
Student Name:	**Dr. Santosh Kumar V**
Guide:	**Dr. Nitin Kumar Bajpai**
Co-Guide:	**Dr. Manish Chaturvedy, Dr. Mithu Banerjee, Dr. Archana Bajpayee, Dr. Abhishek Purohit & Dr. Akhil Danesh Goel**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Pravin Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com