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Serum level of hepcidin in chronic kidney disease - A hospital-based study from South India

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ABSTRACT

Background: The term "chronic kidney disease (CKD)" refers to a variety of pathophysiologic conditions characterized by impaired kidney function and a steady fall in glomerular filtration rate. CKD is a slowly progressive silent epidemic that affects approximately 12% of the world population. **Aims and Objectives:** The aim of the study was to estimate the level of serum Hepcidin in patients with CKD compared to healthy controls. **Materials and Methods:** Hundred subjects both male and female over the age of 18 were included in the study and informed consent was obtained from each of them. The study population was divided equally into two groups. Blood serum was collected to estimate level of hepcidin, urea, creatinine, ferritin, iron, total iron binding capacity, hs C-reactive protein (hsCRP), and total proteins. Urine was collected in a sterile container for urine protein creatinine ratio. **Results:** Hepcidin could be a prognostic marker in the clinical outcome of CKD especially in the progression of CKD. There was a highly significant positive correlation of serum Hepcidin with hsCRP, ferritin, creatinine, urea, urine protein creatinine ratio, and erythrocyte sedimentation rate. Significant negative correlation of serum Hepcidin with Creatinine Clearance, Iron, TIBC, Transferrin saturation, Hemoglobin, and Total Proteins had been observed. **Conclusion:** The current study showed that patients with CKD and anemia have considerably higher serum hepcidin concentrations. The level of hepcidin increases as disease progresses in CKD patients. Hepcidin, mediator of anemia in CKD is the potential target to treat anemia in them to prevent death due to cardiovascular complications and improve their quality of life.

Key words: Chronic kidney disease; Hepcidin; Anemia; Blood serum

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INTRODUCTION

The term "chronic kidney disease (CKD)" refers to a variety of pathophysiologic conditions characterized by impaired kidney function and a steady fall in glomerular filtration rate (GFR).[1] Regardless of the underlying etiology, it is characterized as either kidney damage or GFR <60 ml/min/1.73 m^2 for 3 months or more. CKD is a slowly progressive silent epidemic that affects approximately 12% of the world population. Approximately 1.8 million people affected are treated with renal replacement therapy. Diabetes mellitus, hypertension, glomerulonephritis, polycystic kidney disease, urinary tract infection, autoimmune illnesses, kidney stones, and the toxic effects of several medications are the main risk factors for CKD.

CKD is a chronic inflammatory disease. Anemia in CKD is classified under "Anemia of Chronic Disease" and also under "Anemia of Chronic inflammation." Anemia is the most common complication of CKD and an important cause of sudden cardiac death. Anemia in CKD may be due to absolute or relative erythropoietin deficiency, iron deficiency, decreased life span of red blood cells due to uremic toxins, decreased resistance to oxidative stress and malnutrition.[2] Need to detect and correct anemia becomes absolute essential to improve the quality of life and to

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prevent cardiovascular death due to volume overload in CKD patients.

Increased serum levels of cytokines particularly IL-1, IL-6, and TNF-α have a mechanistic link in the pathophysiology of anemia by

1. Decreasing the erythrocyte survival rate
2. Reduced availability of iron due to an increase in hepcidin
3. Direct inhibition of growth of hematopoietic progenitor cells
4. Lowered Erythropoietin production and inadequate Erythropoietin response to anemia.

Sideropenia, despite having an abundance of iron reserves, is the key characteristic of anemia of chronic inflammation.[3] This is reflected by low serum Iron, hypoproliferative marrow, decreased Transferrin saturation in the range of 15–20% and increased serum ferritin.[1]

Serum hepcidin, 25 amino acid antimicrobial peptide synthesized by liver is an acute phase reactant. It binds to the iron export channel ferroportin resulting in phosphorylation, internalization and degradation of ferroportin in the intestinal cells and macrophages resulting in functional or relative iron deficiency.[4]

Hepcidin production in humans is known to be regulated by hypoxia, erythropoiesis/anemia, and iron status.[5] In addition, novel regulators of hepcidin production are still being discovered, such as Vitamin D, which has recently been demonstrated to inhibit HAMP expression *in vitro* and lower levels of circulating hepcidin in healthy volunteers.[6] It is also widely known that inflammation, and more specifically the inflammatory cytokine IL-6, stimulate the production of hepcidin. Hepcidin and C-reactive protein (CRP) or other inflammatory markers have not always been linked, even though heightened CRP levels have been linked to increased hepcidin in some CKD patients. Adults with CKD are frequently thought to have pro-inflammatory conditions.[7,8] In the CKD in children cohort study, there is neither a particularly high prevalence of elevated CRP levels, nor does CRP seem to increase with decreased GFR as had been shown in adults.[9] However, impaired GFR is generally present in this group; therefore, it is unclear which of these potential pathways may be more strongly associated with elevated hepcidin levels.

Thus, in this investigation, serum levels of hepcidin in CKD patients were determined, and the associations between hepcidin, inflammation, and iron metabolism abnormalities were examined.

Aims and objectives

The aim of the study was to estimate the level of serum hepcidin in patients with CKD compared to healthy controls.

MATERIALS AND METHODS

This study was carried out in the Department of Biochemistry, Dhanalakshmi Srinivasan Medical College and Hospital, Perambalur, from October 2020 to August 2022 after receiving consent from the institutional ethics committee.

Hundred subjects both male and female over the age of 18 were included in the study and informed consent was obtained from each of them. The study group comprises of 50 patients with CKD (25 males and 25 females), while the control group consists of 50 healthy people (25 females and 25 males).

Inclusion criteria

The following criteria were included in the study:

1. Patients with CKD with duration from 1 to 10 years.
2. Age >18 years

Exclusion criteria

The following criteria were excluded from the study:

1. Acute and chronic inflammatory disorders
2. Acute kidney injury
3. Nephrotic syndrome
4. Malignancy
5. Patients on immunosuppressive treatment
6. Thyroid disorders

Sample collection

Before the study, informed consent was obtained from each subject. They were given blood samples to take following a 12-h overnight fast. Each subject had a 6 cc fasting venous sample taken under stringent aseptic conditions. To estimate the erythrocyte sedimentation rate (ESR), 1.6 ml of blood are combined with 0.4 ml of anticoagulants. Allow the remaining blood sample to coagulate. The samples were centrifuged at 1000 rpm for 10 min to separate the serum after the clot was retracted.

To estimate the level of hepcidin, an aliquot of the serum was collected and kept at −20°C in the deep freezer. The remaining serum was used to estimate urea, creatinine, ferritin, iron, total iron binding capacity, hsCRP, and total proteins. Urine was collected in a sterile container for urine protein creatinine ratio.

Analysis of blood samples

The following parameters for the serum sample were estimated.

Estimated parameters
1. Hepcidin-Enzyme linked immunosorbent assay
2. hsCRP – Turbidimetric immunoassay
3. Ferritin
4. Iron
5. Total iron binding capacity
6. Urea –Urease (GLDH) method
7. Creatinine-Modified Jaffe's method
8. Total Proteins-Biuret method

Blood samples collected were utilized for the estimation of hemoglobin and ESR.

Calculated parameters
1. Creatinine clearance (C_{cr}) was calculated using Cockcroft-Gault formula

For Males,

$$C_{cr} = \frac{\left[\text{üüüüüü§üü}(\text{üü} \quad)\right] \times \qquad (\quad)}{72 \times \text{ S. creatinine } (\text{mg / dl})}$$

For Females,

$$C_{cr} = \frac{\left[140 - \text{age } (\text{years})\right] \times \text{ weight } (\text{kg}) \times 0.85}{72 \times \text{S. creatinine } (\text{mg / dl})}$$

2. Transferrin saturation

$$\frac{\text{S. iron } \times 100}{\text{total iron binding capacity}}$$

Statistical analysis

Using the Student's t-test and the Chi-square test, the data were statistically analyzed. The data were expressed using the mean and standard deviation. Significant results were defined as "P=0.05." The correlation between the measured values was assessed using the Pearson's correlation coefficient.

Table 1: Age distribution of the study group

Groups	Age (years)		Statistical inference
	Mean	SD	
Control (n=50)	44.72	7.667	T=4.316
Study (n=50)	52.46	10.100	P=0.0001

RESULTS

For this study, 100 participants were chosen, including 50 CKD cases and 50 healthy controls (Table 1). Levels of serum hepcidin, creatinine, urea, hsCRP, iron, ferritin, total iron binding capacity, transferrin saturation, total proteins, and urine protein creatinine ratio were estimated for all the samples. Transferrin saturation and creatinine clearance were calculated from the formulas. Hemoglobin and ESR were estimated.

The age range of the cases was 30 −°70, with a mean age of 54.46. The average age of the controls, who ranged in age from 30 −°65, was 44.72 years. Age and gender differences between the two groups are statistically significant (P=0.05) (Table 2).

The study group's mean level of hepcidin was 17.34 ng/mL, which was considerably higher than the control group's mean level (5.36 ng/mL; P0.0001). In all of the included age categories, serum hepcidin levels were noticeably greater in the study group than the controls (Table 3).

The table below shows the age matched analysis of serum hepcidin between the control and the study group (Table 4).

The mean level in controls (Males: 6.00 ng/mL; Females: 4.72 ng/mL) and the study group (Males: 16.08 ng/mL; Females: 18.60 ng/mL) is compared. Serum hepcidin levels are significantly higher in the study group than in the controls (P=0.0001). Serum hepcidin levels in the study group varied significantly between the sexes (P=0.038) (Table 5).

Hepcidin study group level (T=2.130, P=0.038)

The mean value of hsCRP in the study group 11.28 mg/L and in the control group 4.57 mg/L and the mean value of ferritin levels in the study group is 638.6 μg/L and in the control group is 128.62 μg/L. The values were significantly higher in the cases than in the controls (P<0.0001, statistically significant). Serum iron, total iron binding capacity, transferrin saturation, and hemoglobin levels in the study group were significantly lower when compared to controls (P<0.0001). There was a significantly higher ESR values in the cases (mean 59.8±22.5 mm/h) when compared to controls (mean 10.8±3.1 mm/h) and the P is statistically significant. Higher serum creatinine, blood

Table 2: Age and gender matched analysis between the control and study group

Age (years)	Control (n=50)		Study (n=50)		Statistical inference
	Male	Female	Male	Female	
30–40	5 (20%)	10 (40%)	5 (20%)	4 (16%)	X^2=24.842
41–50	10 (40%)	14 (56%)	6 (24%)	4 (16%)	P=0.003
51–60	8 (32%)	1 (4%)	10 (40%)	12 (48%)	
61–70	2 (8%)	0	4 (16%)	5 (20%)	
Total	25 (100%)	25 (100%)	25 (100%)	25 (100%)	

Table 3: Comparison of serum hepcidin levels between the control and study groups

Groups	Hepcidin (ng/mL)		Statistical inference
	Mean	SD	
Control (n=50)	5.36	2.183	T=17.465
Study (n=50)	17.34	4.331	P=0.0001

Table 4: Age-matched analysis of serum hepcidin between the control and the study group

Age	Hepcidin (ng/mL)			Statistical inference
	Groups	Mean	SD	
30–40 years	Control (n=15)	5.13	2.232	T=8.481
	Study (n=9)	16.78	4.522	P=0.0001
41–50 years	Control (n=24)	4.83	2.014	T=12.003
	Study (n=10)	15.60	3.134	P=0.0001
51–60 years	Control (n=9)	6.44	1.878	T=7.417
	Study (n=22)	17.77	4.385	P=0.0001
61–70 years	Control (n=2)	8.50	2.121	T=2.709
	Study (n=9)	18.78	5.094	P=0.024

Table 5: Gender-matched comparison of serum hepcidin levels between the control and study group

Gender	Groups	S. Hepcidin (ng/mL)		Statistical inference
		Mean	SD	
Male	Control (n=25)	6.00	2.121	T=10.511
	Study (n=25)	16.08	4.300	P=0.0001
Female	Control (n=25)	4.72	2.092	T=15.189
	Study (n=25)	18.60	4.062	P=0.0001

urea, urine protein creatinine ratio, and total proteins values had been observed in the cases than controls (P<0.0001). Creatinine clearance was significantly lower in the cases when compared to controls (P<0.0001) (Table 6).

The table below shows the comparisons of various parameters in the study group in relation to creatinine clearance (Table 7).

There was a highly significant positive correlation of serum hepcidin with hsCRP, ferritin, creatinine, urea, urine protein creatinine ratio, and ESR. Significant negative correlation of serum hepcidin with creatinine clearance, iron, TIBC, transferrin saturation, hemoglobin, and total proteins had been observed (Table 8).

DISCUSSION

Normochromic normocytic anemia is commonly present in patients undergoing predialysis and has a reported incidence of 47.7%.[10] Anemia is a significant and frequent CKD consequence that accelerates the disease's course,

Table 6: Comparisons of various parameters between the control and study group

Parameters	Groups	Mean	SD	Statistical inference
hsCRP (mg/L)	Control (n=50)	4.57	1.60	T=11.527
	Study (n=50)	11.28	3.79	P=0.000
Ferritin (µg/L)	Control (n=50)	128.62	60.59	T=21.051
	Study (n=50)	638.60	160.22	P=0.0001
S. Iron (µg/dl)	Control (n=50)	103.3400	22.10883	T=16.616
	Study (n=50)	37.91	13.16	P=0.0001
TIBC (µg/dl)	Control (n=50)	310.64	31.974	T=16.103
	Study (n=50)	202.4	13.16	P=0.0001
Tr. Sat (%)	Control (n=50)	33.8024	8.70278	T=8.961
	Study (n=50)	19.16	6.33	P=0.0001
Hb (g/dl)	Control (n=50)	12.6680	0.66714	T=-31.111
	Study (n=50)	7.8560	0.86665	P=0.0001
ESR (mm/hr)	Control (n=50)	10.80	3.104	T=15.253
	Study (n=50)	59.82	22.511	P=0.0001
Creatinine (mg/dl)	Control (n=50)	0.692	0.082	T=12.481
	Study (n=50)	5.30	2.56	P=0.0001
Creatinine clearance (ml/min)	Control (n=50)	120	12.5	T=42.640
	Study (n=50)	19.60	11.36	P=0.0001
Urea (mg/dl)	Control (n=50)	22.42	5.85	T=21.555
	Study (n=50)	118.3	28.9	P=0.0001
Urine protein Creatinine Ratio	Control (n=50)	0.120	0.048	T=18.844
	Study (n=50)	1.06	0.312	P=0.0001

causes cardiovascular problems, and raises morbidity and mortality rates in CKD patients. Renal anemia is regarded as a unique type of inflammatory anemia.[11] Hepcidin levels are controlled by erythropoiesis and iron status; they are decreased by anemia and hypoxia and elevated by inflammation and iron-rich dietary intake without overload.[12] Hepcidin appears to be suppressed, which may be related to the rise in erythropoiesis brought on by the disease's distinctive anemia and, as a result, increase intestinal iron absorption.[13]

CKD is a chronic inflammatory state where there is elevation of inflammatory cytokines like IL-6, TNF-α, IL-1, IFN-γ, and acute phase reactants such as hepcidin, hsCRP, and Ferritin. Despite of improvement in treatment modalities such as dialysis over the past decades, the morbidity and mortality in CKD still remains high. Recent studies point out that chronic inflammation is the major contributor of the mortality and morbidity in CKD.

Elevation of inflammatory markers is associated with most of the complications in CKD like anemia and atherosclerotic cardiovascular diseases. The cause for anemia of CKD is multifactorial and it is due to uremia, uremic toxins, inflammatory cytokines, and their decreased clearance. These inflammatory cytokines (IL-6, TNFα) are also responsible for upregulation of HAMP gene and hepcidin synthesis.

Table 7: Comparisons of various parameters in the study group in relation to creatinine clearance

Parameters	Creatinine clearance (ml/min)		
	30–59 (n= 11) (Mean±SD)	15–29 (n= 14) (Mean±SD)	<15 (n= 25) (Mean±SD)
Hepcidin (ng/mL)	13.09±1.758	16.93±3.710	19.44±4.073
hsCRP (mg/L)	9.88±2.19	10.47±3.84	12.36±4.11
Ferritin (µg/L)	575.91±175.56	606.64±150.144	684.08±151.032
B. UREA (mg/dl)	115±19.31	117.79±35.936	123.3±27.59
S. CREATININE (mg/dl)	2.4±1.73	3.8357±1.06	7.412±0.433
S. IRON (µg/dl)	46.63±10.29	40.42±19.88	32.68±5.08
TIBC (µg/dl)	202.4±35.75	188.86±37.55	209.96±34.603
Transferrin saturation (%)	23.28±3.84	21.21±7.55	16.19±5.31
Hemoglobin (g/dl)	8.69±0.48	8.11±1.00	7.34±0.50
ESR (mm/hr)	52.36±16.19	54.00±24.33	66.36±22.69
Total proteins (g/dl)	5.78±0.40	5.77±0.30	5.6±0.5
Urine. PCR	0.91±0.17	1.06±0.38	1.14±0.30

Table 8: Pearson's correlation

Hepcidin	correlation value	Statistical inference
AGE	0.177(**)	P<0.01*
Wt	0.100	P>0.01
BP (mmHg) – Sys	0.103(**)	P<0.01*
BP (mmHg) – Dia	0.084(**)	P<0.01*
hsCRP	0.455(**)	P<0.01*
Ferritin	0.417(**)	P<0.01*
Sr.Iron	−0.160(**)	P<0.01*
TIBC	−0.025(**)	P<0.01*
Tr.Sat	−0.157(**)	P<0.01*
Hb	−0.608(**)	P<0.01*
ESR	0.314(**)	P<0.01*
Creat	0.493(**)	P<0.01*
C_{cr}	−0.625(**)	P<0.01*
UREA	0.349(**)	P<0.01*
UR. PCR	0.139(**)	P<0.01*
T.Prot	−0.012(**)	P<0.01*
N	100	

*P<0.01 = Significant

In the current study, 100 participants in all were examined, 50 of them had chronic renal disease, and the remaining 50 served as controls. In our present study, we measured the serum hepcidin concentrations in CKD patients to assess the correlation between inflammation and anemia in CKD. In the study, we found that the concentration of serum hepcidin was found to be significantly increased in patients with CKD (Mean value: 17.34±4.33 ng/mL) when compared to the control group (Mean value: 5.36±2.18 ng/mL; P<0.0001). Other studies found hepcidin levels were noticeably higher in CKD patients compared to controls. In CKD cases, increased levels of serum urea, creatinine, ferritin, and hsCRP were discovered.[14-16]

In this study, serum hepcidin levels were found to be progressively elevated along the spectrum of CKD (from stage 3(C_{cr}= <60 ml/min) to stage 5 (C_{cr}<15 ml/min)). Level of hepcidin increases as renal function declines, which are backed up by a very strong association between serum hepcidin and creatinine in CKD. This study also correlates with the previous studies done by Zaritsky J et al.[15]

The mean level of serum hepcidin in CKD cases is 17.34±4.3 ng/mL. In addition, we found that as compared to controls, serum hepcidin levels were significantly higher in both genders and all age groups, suggesting that age and gender have no bearing on serum hepcidin levels.

Further, Pearson's correlation revealed an inverse relationship between S. Hepcidin and Creatinine Clearance. Since hepcidin is metabolized in kidney and excreted in urine, the concentration of S. Hepcidin increases with deterioration of renal function. An elevated S. Hepcidin level in CKD is due to progression in stages of CKD associated with inflammation and malnutrition.

In this study, hsCRP is significantly elevated in CKD cases when compared with controls (Mean level: Cases- 11.28±3.79 mg/L; Controls - 4.57±1.60 mg/L; P<0.0001). Progressive increase in serum hsCRP is detected as there is a decline in renal function and a linear correlation has been observed between hepcidin and hsCRP (r=0.455, P<0.01). Elevated hsCRP is a risk stratification marker and it is associated with poor outcome in CKD. The previous studies also show a positive association between hsCRP and hepcidin.[17,18]

Ferritin, an acute phase reactant is also elevated in CKD. In this study, the mean level of ferritin in cases (638.6±160.22 µg/L) is found to be increased when compared to controls (128.62±60.59µg/L) and it is statistically significant (P<0.0001). This increase is associated with progressive decline in renal function. Positive correlation is observed between serum hepcidin and ferritin (r=0.417; P<0.01). Elevated S. Ferritin along with hsCRP indicates that there is an acute inflammatory reaction which inhibits the mobilization of iron from its stores. These findings correlate with the previous study done by Wish.[19]

Serum iron is decreased in CKD patients as CKD progresses. The mean level of S. iron in cases (37.9±13.1 µg/dl) is

decreased when compared to controls (103.4±22.1 μg/dl) which is statistically significant (P<0.0001). The decreased level of serum iron in CKD is due to decreased release of iron from intestinal cells or macrophages which is due to inflammation. In Anemia of CKD, inverse relationship exists between S. Iron and Ferritin when compared to Iron deficiency anemia where the relationship is linear. We also observed a negative correlation between S. Iron and hepcidin (r =−0.160, P<0.01).

Total iron binding capacity is found to be significantly reduced in CKD cases (Mean level: cases-202.4±13.6 μg/dl; controls-310.6±31.9μg/dl; P<0.0001). Pearson's correlation revealed a negative relationship between hepcidin and TIBC.

Our study also showed a decrease in transferrin saturation in CKD cases (Mean level: cases – 19.1±6.3%; controls – 33.8±8.7 %; P<0.0001) and also strong significant negative correlation with hepcidin (r=−0.157; P <0.01). This result correlates with the previous studies done by Mehdi and Toto.[20] Similarly, Roy et al.,[16] showed that the mean transferrin saturation was lower in the CKD group (28.454±8.048%) compared to control participants (31.65±5.294%), but that the difference between the two groups was statistically insignificant with a P=0.07 (P>0.05).

The mechanism underlying this is as follows: Hepcidin, an acute phase reactant produced by the liver cause's phosphorylation and internalization of Ferroportin, which prevents release of iron from intestinal cells and macrophages to circulating transferrin and a decrease in transferrin saturation.

In this study, hemoglobin level is decreased in the cases (mean level: cases-7.8±0.86 g/dl; controls-12.6±0.66 g/dl; P<0.0001) and negatively correlated with hepcidin level (r =−0.608; P<0.01). Anemia of CKD is an important cause of cardiovascular mortality where the levels of inflammatory cytokines are increased. Similar study by Roy et al.,[16] found that the mean hemoglobin value in the CKD group was 7.324±1.213 g/dl compared to 13.922±1.758 g/dl in the control group, with a P<0.05. We also observed a significantly higher ESR in cases than controls (Mean level: cases-59.82±22.51 mm/h; and controls-10.8±3.1 mm/hr; P<0.0001). This signifies a strong inflammatory pathology underlying anemia of CKD. Further significant positive correlation exists between ESR and hepcidin (r=0.314; P<0.01). In this study, significant positive correlation exists between urine protein creatinine ratio and hepcidin in cases (r=0.139; P<0.01) which signifies a progressive decline in renal function in this study.

In this study, serum total proteins are decreased in cases when compared to controls (Mean level: cases - 5.6±0.4 g/dl; and controls - 7.2±0.5 g/dl; P<0.0001). It is negatively correlated with S. Hepcidin in study group which signifies that malnutrition and inflammation are associated with the pathogenesis in CKD.

Hence, the results of the present study suggest that hepcidin, an acute phase reactant synthesized by liver, is elevated due to upregulation of HAMP gene by inflammatory cytokines and other positive regulators. Hepcidin causes phosphorylation, internalization, and degradation of ferroportin which is responsible for transport of iron from intestinal cells and macrophages into circulation and hence erythropoiesis. The findings observed in this study showed a close relationship between anemia, inflammation, and malnutrition with progressive decline in renal function.

Limitations of the study

Our study has the following limitations

- Sample size is very small
- Erythropoietin is not measured in this study to demonstrate relative erythropoietin deficiency
- Interleukin-6 is not included in this study
- Transferrin saturation receptor which differentiates Iron Deficiency Anemia from anemia of chronic inflammation is not measured

CONCLUSION

The current study showed that patients with CKD and anemia have considerably higher serum hepcidin concentrations. Chronic inflammation could be responsible for the elevated serum hepcidin either due to increased production of Acute Phase Reactants by liver or decreased clearance of hepcidin by affected kidney. The level of hepcidin increases as disease progresses in CKD patients.

Hepcidin causes phosphorylation, internalization and degradation of ferroportin in intestine and macrophages and alters iron homeostasis in CKD. Hence, iron stored as ferritin is increased. Hepcidin, mediator of anemia in CKD is the potential target to treat anemia in them to prevent death due to cardiovascular complications and improve their quality of life.

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