Journal of Clinical Laboratory Analysis 27: 504–510 (2013)

Hepcidin-25 Concentrations Are Markedly Increased in Patients With Chronic Kidney Disease and Are Inversely Correlated With Estimated Glomerular Filtration Rates

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Background: Hepcidin-25 regulates iron homeostasis by binding the iron transporter ferroportin, causing its degradation. Increased hepcidin-25 causes decreased intestinal iron absorption and release from intracellular stores. Our objective in this study was to measure hepcidin-25 levels in patients with chronic kidney disease (CKD) to determine if they might contribute to the anemia of CKD. *Methods:* We used a hepcidin-25-specific enzyme-linked immunosorbent assay to measure hepcidin-25 in 103 CKD patients and 100 healthy individuals. We assessed in CKD subjects the correlation of hepcidin-25 with creatinine, estimated glomerular filtration rate (eGFR), hemoglobin, blood urea nitrogen, serum iron, transferrin, and ferritin. *Results:* Hepcidin-25 concentrations in CKD patients were significantly increased compared to healthy subjects (60.4 $\pm$ 6.1 μg/l vs. 3.0 $\pm$ 0.5 μg/l, $P < 0.001$). Hepcidin-25 concentrations were directly correlated with creatinine ($R = 0.28$, $P = 0.004$) and inversely correlated with eGFR ($R = -0.32$, $P = 0.001$). Hepcidin-25 levels were also correlated with transferrin ($R = -0.28$, $P = 0.004$) and ferritin ($R = 0.80$, $P < 0.001$). *Conclusion:* The direct correlation of hepcidin-25 with creatinine and its inverse correlation with eGFR suggest that hepcidin-25 levels increase as renal function deteriorates, possibly due to decreased hepcidin-25 renal clearance. J. Clin. Lab. Anal. 27:504–510, 2013.

Key words: anemia; ferritin; hepcidin; immunoassay; iron

INTRODUCTION

Iron homeostasis is essential in sustaining red blood cell production and subsequent oxygen distribution throughout the body. The 25-amino acid active form of hepcidin (hepcidin-25) is the master hormone regulating iron homeostasis in humans (1, 2) Hepcidin-25 is synthesized and secreted by the liver and controls dietary iron absorption and release of hepatic and macrophage iron stores by binding directly to the iron transport protein ferroportin, causing it to be internalized and degraded. This results in decreased iron transport across the cell membrane, which decreases iron availability for hemoglobin production and erythropoiesis (3–7).

In healthy individuals, iron status regulates hepatic hepcidin synthesis and secretion to guard against anemia or iron overload (8, 9). Hepcidin deficiency has been described in hereditary hemochromatosis, a disease in which inappropriately high levels of iron are absorbed and cause iron toxicity (10–12). Hypoxia and anemia reduce serum hepcidin levels in order to facilitate iron transport and erythropoiesis (8). In anemia of chronic disease and inflammation, there is reduced iron availability for erythropoiesis (13–15). Recent data have suggested that there is a significant increase in the hepcidin levels of individuals with chronic kidney disease (CKD) (16–18). Such increases would suggest that elevated hepcidin and its consequential inhibition of iron transport may play a key role in the anemia observed in CKD.

Until recently, the lack of an immunoassay to selectively measure hepcidin-25 in serum has left many unanswered questions around its role in the anemia of chronic

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Received 25 January 2013; Accepted 8 May 2013
DOI 10.1002/jcla.21634
Published online in Wiley Online Library (wileyonlinelibrary.com).

disease, including CKD (19). Previous approaches have included measuring urine hepcidin and the hepcidin precursor, prohepcidin. These analytes, however, function only as surrogate measures of serum hepcidin-25, and their associations with iron status are not well understood (20–24). Specific mass spectrometry assays have been used to measure serum hepcidin-25 concentrations, however, the relatively low throughput and high amount of operator expertise required can be limiting (25). Development of an immunoassay to measure active hepcidin has been difficult due to the size of the peptide (approximately 2.7 kDa), and the fact that it contains four disulfide bonds (26, 27). To add further complexity, there are inactive N-terminal cleavage products of hepcidin-25 (hepcidin-20 and hepcidin-22) that do not appear to play a role in maintaining iron homeostasis (28). There have been reports describing serum hepcidin competitive immunoassays that use polyclonal capture antibodies, but there are inherent concerns around cross-reactivity with hepcidin-20 and hepcidin-22 using this approach (29). Furthermore, recent data demonstrated that these inactive variants may be disproportionately increased in CKD subjects (25). It is therefore important that an assay specific for hepcidin-25 be utilized to assess hepcidin levels in patients with renal disease.

Recently, our laboratory developed a dual monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) that is highly specific for hepcidin-25 in human serum (30). In this study, we utilized our assay to measure serum levels of hepcidin-25 in 103 patients with CKD, and compared them with serum hepcidin-25 levels of 100 healthy subjects to better understand the concentrations of hepcidin-25 present in CKD. We hypothesized that hepcidin-25 levels might be increased in CKD due to the inability of the kidneys to filter and clear hepcidin-25 and that the increased levels might be at least partly responsible for the anemia observed. We then assessed the correlation of hepcidin-25 with creatinine, estimated glomerular filtration rate (eGFR), hemoglobin, blood urea nitrogen (BUN), serum iron, transferrin, and ferritin in CKD patients.

METHODS

Human Samples (CKD Patients)

Human serum samples were collected from 103 individuals with CKD from the IU Methodist Research Institute in Indianapolis, Indiana (ages 28–90 years, average age 68 years). Of the 103 individuals, 56 were males and 47 were females. The group represented CKD stages 3–4 and end-stage renal disease (ESRD, stage 5) without dialysis. The numbers of CKD patients at each particular stage were as follows: stage 5–51 patients, stage 4–23 patients, stage 3–29 patients. The group of CKD patients studied had a diverse range of causes resulting in CKD. These included diabetes mellitus, hypertension, polycystic kidney disease, focal segmental glomerulosclerosis, transplant rejection, and membranous nephropathy. We were unable to obtain iron supplementation data, transfusion histories, or records of erythropoietin (EPO) and other erythropoiesis-stimulating agent (ESA) usage for these patients due to patient privacy restrictions around sample donation. Analysis of creatinine, hemoglobin, and BUN were performed using an I-STAT blood analyzer (Abbott, Abbott Park, North Chicago, IL USA). eGFRs were determined by incorporating creatinine levels and biometric data into the Cockroft–Gault formula (31). Serum iron, transferrin, and ferritin levels were measured in the CKD patients using an automated Roche (Indianapolis, IN USA) Hitachi analyzer. After obtaining protocol approval and proper informed consent from an institutional review board, all samples were collected, stored, and de-identified to protect patient privacy. Samples were shipped on dry ice and stored at -70°C prior to analysis of hepcidin-25.

Human Samples (Healthy Individuals)

Hundred serum samples from healthy volunteers (ages 18–64 years, mean age 37 years) were purchased from Valley Biomedical Inc., (Winchester, VA USA). The samples were obtained from 50 female and 50 male donors. Samples were received on dry ice and stored at -70°C prior to analysis of hepcidin-25 levels.

Hepcidin-25 ELISA

Hepcidin-25 levels were measured as previously described (30) with minor modifications. Briefly, a human hepcidin-25 MesoScale Discovery (MSD) ELISA was performed using streptavidin-coated and blocked wells that were incubated for 1 hr with biotinylated anti-hepcidin-25 capture antibody (2 mg/l). Afterward, wells were aspirated and washed three times with TBST (Tris-buffered saline containing 10 mmol/l Tris pH 7.40, 150 mmol/l NaCl with 1 ml Tween 20/l). Next, 100 μl of hepcidin-25 (Peptides International, Louisville, KY USA) standards (varying concentrations of hepcidin-25 protein in assay buffer consisting of 50 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.40, 150 mmol/l NaCl, 10 ml/l Triton X-100, 5 mmol/l EDTA (ethylenediaminetetraacetic acid), and 5 mmol/l EGTA (ethylene glycol tetraacetic acid)) were added to the wells to generate a standard calibration curve. Serum samples were diluted in a ratio of 1:20 in assay buffer and added to their respective wells, and the ELISA plate was incubated for 1 hr at room temperature. Following

aspiration, wells were washed three times with TBST, and 100 μl of a 1:1,000 dilution of conjugate antibody (ruthenium-labeled antihepcidin detection antibody, 1 mg/l) were added to the wells for a 1 hr incubation at room temperature. Following aspiration, wells were washed three times with TBST, and the plate was developed using a MSD reader, which recorded ruthenium electrochemiluminescence.

Data Analysis

MSD software and SigmaPlot version 8.0 were used for fitting ELISA calibration curves. For each group of subjects studied, results were expressed as the mean ± SEM. Data were plotted and graphed using version 2.98 of the program FigP (Biosoft). Comparisons of hepcidin-25 levels with other analytes were performed using the same program. Data were analyzed by one-way analysis of variance followed by comparisons between the means using the least significant difference test. For each correlation performed between independent analytes, statistical analysis was performed using the same program to calculate Spearman correlation coefficients. In each case, a *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

In our current study, we utilized our previously described, sensitive and specific ELISA to assess serum hepcidin-25 levels in patients with CKD. Routine laboratory profiles for these patients are shown in Table 1. In addition, we analyzed the sera of 100 healthy individuals in which the mean serum hepcidin-25 level was 3.0 ± 0.5 μg/l, consistent with the concentrations of hepcidin-25 that our laboratory has previously observed in healthy volunteer subjects (30).

As Figure 1 demonstrates, hepcidin-25 levels were approximately 20-fold elevated in patients with CKD compared to healthy subjects. This marked increase in mean serum hepcidin-25 concentrations in the CKD patients compared to healthy subjects was highly significant

TABLE 1. CKD Patients' Profiles—Results Are Shown as the Mean ± SEM for 103 CKD Patients

Analyte	Value
Cr	4.9 ± 0.3 mg/dl
eGFR	22.0 ± 1.9 ml/min
BUN	39.8 ± 2.2 mg/dl
Hemoglobin	12.6 ± 0.8 g/dl
Serum iron	69.4 ± 4.4 μg/dl
Transferrin	1.9 ± 0.1 g/l
Ferritin	671.7 ± 57.5 μg/l

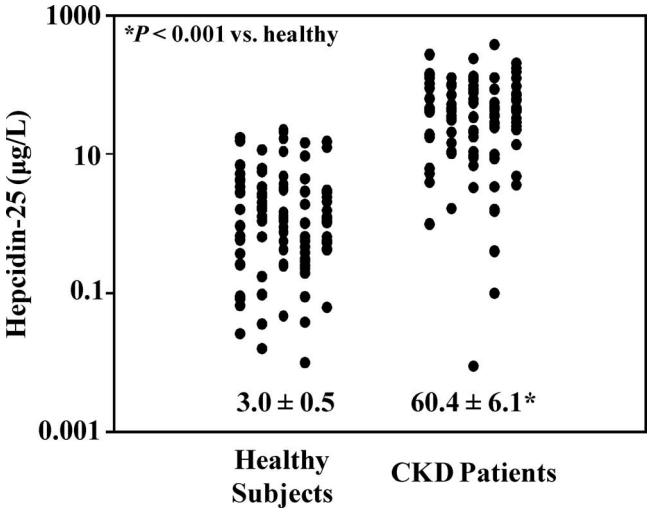

Fig. 1. Serum hepcidin-25 concentrations in CKD patients and normal subjects—serum samples from 100 healthy subjects and 103 patients with CKD were analyzed for hepcidin-25 concentrations. Results are plotted as individual points with the mean ± SEM also shown. Serum hepcidin-25 concentrations in patients with CKD were significantly increased compared to healthy subjects (60.4 ± 6.1 μg/l vs. 3.0 ± 0.5 μg/l, $P < 0.001$).

(60.4 ± 6.1 μg/l vs. 3.0 ± 0.5 μg/l, $P < 0.001$). These results are in agreement with recent findings that have reported increases in total hepcidin for patients with CKD (16, 17, 32). Our current results further indicate that concentrations of the active form of the hormone (hepcidin-25) are dramatically elevated in CKD.

After obtaining these data, we next examined the correlation of hepcidin-25 with creatinine, eGFR, BUN, hemoglobin, serum iron, transferrin, and ferritin in the patients with CKD. Creatinine levels were first analyzed to assess the status of renal function in the CKD patients and were compared to hepcidin-25 concentrations. As expected, creatinine levels were markedly increased in serum samples from CKD patients. As shown in Figure 2A, creatinine concentrations were significantly and directly correlated with hepcidin-25 levels in CKD patients ($R = 0.28$, $P = 0.004$). Creatinine levels were then incorporated along with biometric data into the Cockroft–Gault equation for determining the eGFR (31) in CKD patients. The eGFR was then plotted against hepcidin-25 concentrations in the CKD patients (Fig. 2B). As Figure 2B shows, there was a significant inverse correlation between serum hepcidin-25 concentrations and eGFR in the CKD patients ($R = -0.32$, $P = 0.001$), indicating that hepcidin-25 levels tended to increase as the eGFR decreased.

One limitation of our current study is that we were unable to obtain iron supplementation data, transfusion histories, or records of ESA usage due to patient privacy restrictions around sample donation. It should be remembered, however, that iron supplementation, transfusions,

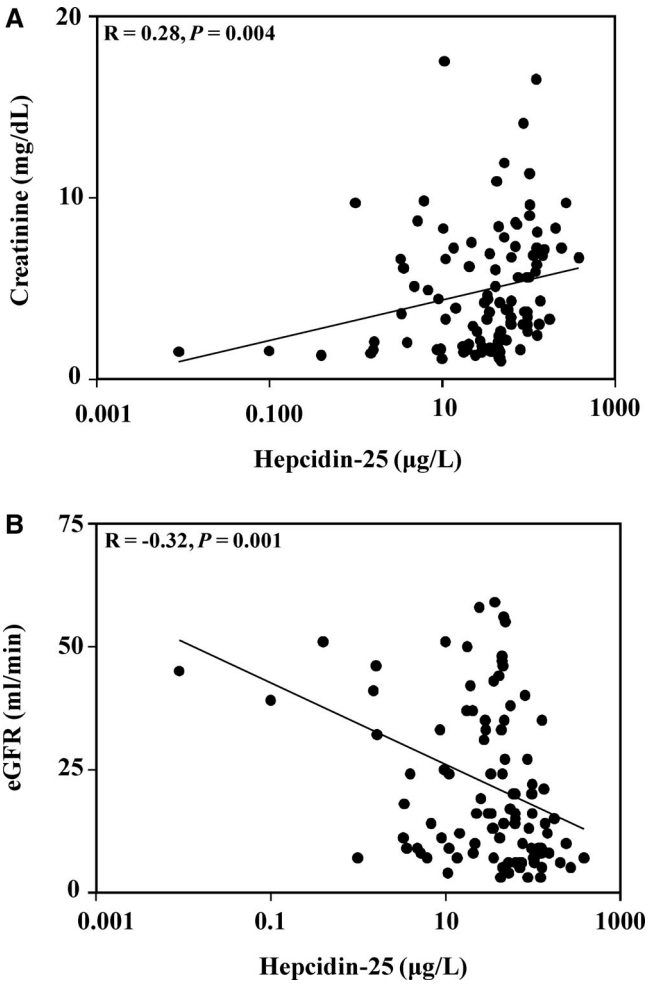

Fig. 2. Relationship between hepcidin-25, creatinine, and eGFR in patients with CKD. (A) Serum hepcidin-25 and creatinine concentrations were compared in CKD patients. Hepcidin-25 concentrations were directly correlated with creatinine levels ($R = 0.28$, $P = 0.004$). (B) Serum hepcidin-25 concentrations and eGFR were compared in CKD patients. The two measures were significantly inversely correlated in the CKD patients ($R = -0.32$, $P = 0.001$).

and ESAs would all be expected to decrease hepcidin levels, which if anything should decrease the correlation of hepcidin-25 to creatinine and eGFR. In spite of this limitation, we were still able to demonstrate significant correlations between hepcidin-25 and eGFR and creatinine in the CKD patients.

In contrast, there was no significant correlation between hepcidin-25 concentrations and BUN levels in the CKD patients ($R = 0.08$, $P = 0.39$). These results were not surprising, given that BUN can be influenced by multiple factors in CKD including altered tubular urea reabsorption and is, thus not a standard marker in the estimation of CKD stage.

We also analyzed the association between serum hepcidin-25 levels and the hemoglobin concentration in

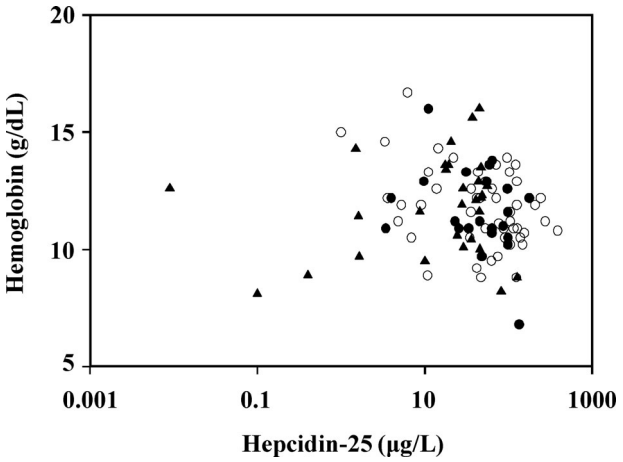

Fig. 3. Comparison of hepcidin-25 and hemoglobin in patients with CKD—serum hepcidin-25 and blood hemoglobin concentrations were examined in patients with CKD. Results are plotted for patients with stage 5 CKD (open circles), stage 4 CKD (solid circles), and stage 3 CKD (solid triangles). There was no significant correlation between hepcidin-25 and hemoglobin for any CKD stage ($R = -0.14$, $P = 0.32$ for stage 5; $R = -0.33$, $P = 0.11$ for stage 4; $R = -0.12$, $P = 0.52$ for stage 3).

patients with CKD. Figure 3 shows the relationship between hepcidin-25 levels and hemoglobin concentrations in the CKD patients and demonstrates that there was no significant correlation between hepcidin-25 concentrations and hemoglobin levels in patients regardless of CKD stage. It should be noted, however, that we were limited in our knowledge of CKD patient transfusion history, recombinant EPO (rhEPO) usage, and the use of other ESA treatments. Since both ESA usage as well as red cell transfusions would be expected to increase hemoglobin levels while lowering hepcidin-25 concentrations (16, 33, 34), and thus disrupt any correlation between the two, this may explain why there was no significant correlation between hepcidin-25 and hemoglobin concentrations in the CKD patients.

We next compared hepcidin-25 concentrations in the CKD patients to serum iron levels, transferrin, and ferritin. In the case of serum iron, as Figure 4A demonstrates, there was no significant correlation with hepcidin-25 concentrations ($R = 0.10$, $P = 0.31$). In contrast, as shown in Figure 4B, hepcidin-25 concentrations were inversely correlated with serum transferrin levels ($R = -0.28$, $P = 0.004$). As Figure 4C demonstrates, ferritin levels were markedly increased in patients with CKD, and hepcidin-25 concentrations were highly and directly correlated with the ferritin values ($R = 0.80$, $P < 0.001$).

Finally, we investigated the relationship between age and hepcidin-25 concentrations. Since most of the normal subjects that we measured hepcidin-25 levels in were younger than the CKD patients, we wanted to rule out the possibility that a high correlation between hepcidin-25 values and age could account for the large

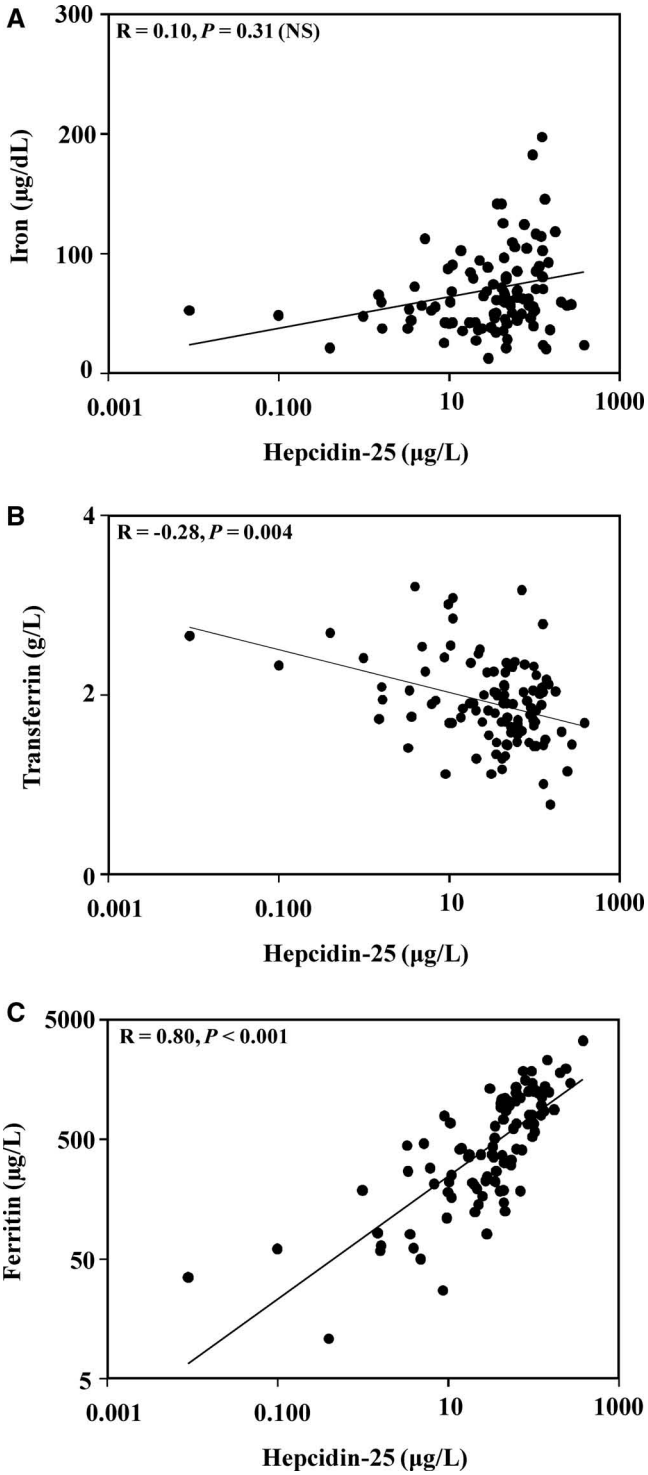

Fig. 4. Relationship between hepcidin-25 and iron, transferrin, and ferritin in patients with CKD. (A) Hepcidin-25 and serum iron levels in CKD patients were compared. There was no significant correlation between the two ($R = 0.10$, $P = 0.31$). (B) Hepcidin-25 and transferrin levels in CKD patients were compared. The two analytes were inversely correlated ($R = -0.28$, $P = 0.004$). (C) Hepcidin-25 and serum ferritin levels in CKD patients were compared. Hepcidin-25 was directly correlated with serum ferritin concentrations ($R = 0.80$, $P < 0.001$).

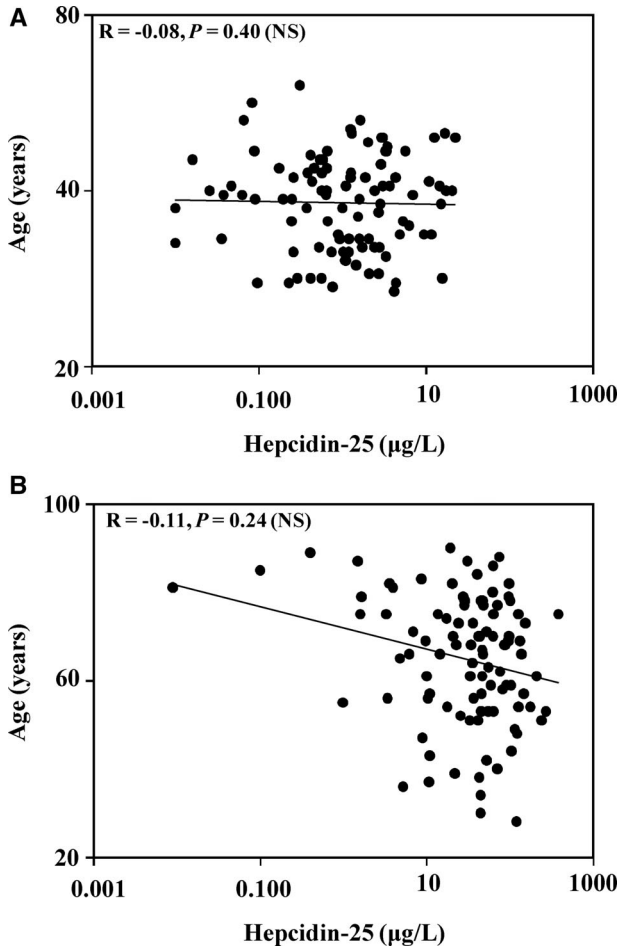

Fig. 5. Comparison of age and hepcidin-25 concentrations in normal subjects and in patients with CKD. (A) Hepcidin-25 and age were compared in the normal subjects. There was no significant correlation between the two ($R = -0.08$, $P = 0.40$). (B) Hepcidin-25 and age in CKD patients were compared. There was no significant correlation between the two ($R = -0.11$, $P = 0.24$).

difference in hepcidin-25 levels observed in the CKD patients vs. the normal subjects. As Figure 5A shows, there was no significant correlation between hepcidin-25 concentrations and age in the normal subjects ($R = -0.08$, $P = 0.40$). Similarly, in the patients with CKD, there was also no significant correlation between age and hepcidin-25 values ($R = -0.11$, $P = 0.24$), suggesting that the increased age of the patients with CKD did not account for the large increases in hepcidin-25 levels compared to normal subjects.

DISCUSSION

In healthy individuals, diminished iron, hypoxia, and anemia decrease hepatic hepcidin synthesis and secretion in order to allow for sufficient iron export into the plasma to support erythropoiesis in the bone marrow (8).

In CKD, however, patients often present with anemia also appear to have decreased erythropoiesis (2, 8). The results of our current study indicate that circulating concentrations of hepcidin-25, the active form of the hormone, are markedly elevated in patients with CKD.

Our current data are consistent with similar findings using serum total hepcidin including competitive plasma hepcidin ELISA methods that utilize polyclonal hepcidin capture antibodies (16, 17, 35, 36). The importance of our current findings are highlighted by the fact that levels of inactive forms of hepcidin, such as hepcidin-20 and hepcidin-22, have been reported to be disproportionately increased in CKD, which can make data interpretation from immunoassays that react with multiple forms of hepcidin difficult (37). In contrast, our current data, which were generated using a specific sandwich assay for hepcidin-25 (30), show definitively that the active form of the hormone is markedly increased in CKD.

Creatinine is an established marker for determining renal function and GFR (31, 38). Within our CKD subject group, hepcidin-25 concentrations showed significant direct correlation with creatinine and significant inverse correlation to eGFR. Our observations indicating that hepcidin-25 levels were correlated with creatinine and eGFR differ somewhat from those of Peters et al., who previously reported that serum hepcidin-25 levels in patients with CKD were independent of GFR (18). It is unclear why our results were different from those of Peters et al., but one possibility may be that the populations of CKD patients differed in the respective studies.

Contrary to the significant correlations with creatinine and eGFR, we did not observe any significant correlation between hepcidin-25 and BUN in the CKD patients. Given the variables that can impact BUN, the lack of correlation with hepcidin-25 is not surprising. Since urea concentrations can be influenced by several factors, including tubular urea reabsorption, BUN is typically not considered as reliable a marker for assessing renal function as creatinine (31, 38).

Recent reports have suggested a modest and significant correlation between hepcidin and hemoglobin (39, 40). While the samples from our CKD subject group collectively displayed low hemoglobin levels, we did not observe a significant correlation. Given that we did not have extensive information around patient transfusion, ESA usage, or intravenous iron administration, further studies would be necessary to better examine this relationship.

With regard to the correlation of hepcidin-25 to other markers of iron metabolism, we found that hepcidin-25 concentrations did not correlate with serum iron, were modestly correlated with transferrin, and highly correlated with serum ferritin levels, which themselves were markedly elevated in the CKD patients. The observation that ferritin levels are elevated in CKD patients and correlate with hepcidin-25 levels suggests that the increased ferritin levels may be driving the increases in hepcidin-25. Alternatively, it is also possible that the inability of the kidneys to clear hepcidin-25 results in increases in circulating hepcidin-25 that prevent iron export from tissues via ferroportin, thus resulting in increased tissue and ultimately serum ferritin.

In the present study, we have shown that CKD patients have markedly elevated hepcidin-25 levels that are significantly correlated with well-established markers of renal function including serum creatinine and eGFR. Further studies are clearly warranted to better understand the relationship between hepcidin-25, iron status, red cell indices, and erythropoiesis in patients with diminished renal function.

ACKNOWLEDGMENTS

The authors thank Mark Willey for his technical assistance.

ABBREVIATIONS

BUN = blood urea nitrogen
CKD = chronic kidney disease
ELISA = enzyme-linked immunosorbent assay
ESRD = end-stage renal disease
EPO = erythropoietin
ESA = erythropoiesis-stimulating agent
eGFR = estimated glomerular filtration rate
MSD = MesoScale Discovery
rhEPO = recombinant human erythropoietin
TBST = Tris-buffered saline plus Tween

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