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Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease

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Hepcidin is a critical inhibitor of iron export from macrophages, enterocytes, and hepatocytes. Given that it is filtered and degraded by the kidney, its elevated levels in renal failure have been suggested to play a role in the disordered iron metabolism of uremia, including erythropoietin resistance. Here, we used a novel radioimmunoassay for hepcidin-25, the active form of the hormone, to measure its levels in renal disease. There was a significant diurnal variation of hepcidin and a strong correlation to ferritin levels in normal volunteers. In 44 patients with mild to moderate kidney disease, hepcidin levels were significantly elevated, positively correlated with ferritin but inversely correlated with the estimated glomerular filtration rate. In 94 stable hemodialysis patients, hepcidin levels were also significantly elevated, but this did not correlate with interleukin-6 levels, suggesting that increased hepcidin was not due to a general inflammatory state. Elevated hepcidin was associated with anemia, but, intriguingly, the erythropoietin dose was negatively correlated with hepcidin, suggesting that erythropoietin suppresses hepcidin levels. This was confirmed in 7 patients when hepcidin levels significantly decreased after initiation of erythropoietin treatment. Our results show that hepcidin is elevated in renal disease and suggest that higher hepcidin levels do not predict increased erythropoietin requirements.

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Replacement of erythropoietin (EPO) is the mainstay of treatment of anemia in renal failure, reflecting the importance of renally produced EPO in regulating hematocrit. However, it is now recognized that sensitivity to EPO therapy in end-stage renal disease is often limited by a functional defect in iron availability, and in most patients intravenous iron therapy improves the response.[1] Resistance to EPO has been associated with increased mortality in renal disease,[2] and there are concerns that regular intravenous iron administration may have adverse consequences.[3,4] It is therefore hoped that a better understanding of abnormal iron homeostasis in renal disease would permit therapeutic improvements increasing EPO responsiveness.

Hepcidin is the key hormone governing mammalian iron homeostasis. It is produced predominantly in the liver and undergoes furin-mediated cleavage to produce the 25-amino-acid active form (hepcidin-25).[5] Hepcidin causes degradation of the iron export protein ferroportin, which is expressed by macrophages, enterocytes, and hepatocytes, thereby preventing iron export from these cells into the plasma.[6] Hepcidin is regulated by iron intake[7] and anemia,[8] and its deficiency underlies hereditary hemochromatosis in humans[9] and causes iron overload in mouse models.[10]

N-terminal cleavage of hepcidin-25 produces the biologically less active hepcidin-22 and hepcidin-20 peptides[11,12] that, together with hepcidin-25, are excreted in the urine.[12,13] Recent work has suggested that hepcidin-25, like β2-microglobulin, is freely filtered and reabsorbed in the proximal tubule.[14] In this context, it has been suggested that the functional iron deficit that accompanies renal disease may be attributable to increased circulating levels of hepcidin due to impaired urinary excretion.[15]

Hepcidin expression is also induced by inflammatory stimuli such as interleukin-6 (IL-6),[16] and because the erythropoietic demand for iron is large in relation to the quantity normally available in the plasma,[12,17] it is believed that excess hepcidin in inflammatory states reduces the iron recycling that is necessary for erythropoiesis, leading to the anemia of chronic disease, which is characterized by reduced erythropoiesis and reduced serum iron despite apparently

normal stores.[18,19] These features are similar to EPO resistance in kidney patients,[20,21] and an important hypothesis is that increased hepcidin causes, and could be a marker of, EPO resistance.

To date, measurement of hepcidin in humans has proved difficult—previous studies measured circulating prohepcidin, levels of which do not appear to be physiologically relevant,[22] or urinary hepcidin using a semiquantitative dot-blot assay.[23] More recently, accurate mass spectrometry-based techniques have been developed.[24] We have developed a specific immunoassay for plasma hepcidin, and report measurement of circulating hepcidin in a large group of renal patients and healthy controls.

RESULTS

Plasma hepcidin levels in 64 healthy controls (34 men) showed a log-normal distribution, with median 10.8 ng/ml and normal range 2–55 ng/ml (5th–95th percentiles). As predicted, log hepcidin was strongly correlated with log ferritin ($R = 0.673$, $P < 0.0001$). Hepcidin levels were higher in men, although this difference was entirely accounted for by the variation in ferritin.

Because iron and EPO levels show a diurnal variation in normal people,[25] we measured levels in eight healthy male controls consuming their usual diet over a 24 h period (Figure 1a). This suggested a diurnal pattern with peak levels in the afternoon, although a flattened profile was observed in the two subjects with lowest ferritin levels (28 and 33 ng/ml, respectively, others >70 ng/ml). These results were confirmed by analysis of paired samples in a further group of 18 controls (8 men; Figure 1b), in whom plasma hepcidin at 1600 hours was approximately twice the level at 0800 hours (mean ratio 2.17, $P = 0.0001$).

We next examined the effect of reduced renal clearance by measuring plasma hepcidin in 44 patients with chronic kidney disease not requiring dialysis (CKD group; age 24–92), and 94 stable hemodialysis patients (HD group; age 28–87; Figure 2). The CKD group had varying degrees of renal impairment (estimated glomerular filtration rate (eGFR) 8–98 ml/min), and none were receiving EPO or intravenous iron therapy, which were administered to the HD group, aimed at maintaining hemoglobin >11 g/100 ml and ferritin >400 ng/ml. Hepcidin was log-normally distributed in both groups: the CKD group exhibited elevated levels compared to controls, with median 26.5 ng/ml (5th–95th percentiles: 3.1–153 ng/ml, $P < 0.001$); and the HD group had significantly higher levels, with median 58.5 ng/ml (5th–95th percentiles: 27.6–158 ng/ml, $P < 0.001$). Moreover, hepcidin was inversely correlated with eGFR in the CKD group ($R = -0.530$, $P = 0.0002$).

To confirm that the elevated measurements in renal disease were not purely due to the accumulation of degradation products, we performed the assay on pooled plasma from control and HD patients, separated by high-performance liquid chromatography (HPLC; Figure 3). Although a small additional peak was detected in HD plasma, over 90% of the signal corresponded chromatographically to hepcidin-25.

We next investigated diurnal variation of hepcidin in CKD and HD patients, and hepcidin clearance during dialysis. Morning (0800 hours) and afternoon (1600 hours) levels were measured in four CKD patients (eGFR 12–28) and six HD patients on a non-dialysis day (Figure 1b), showing no clear diurnal pattern (65.5 vs 65.5 ng/ml in CKD group and

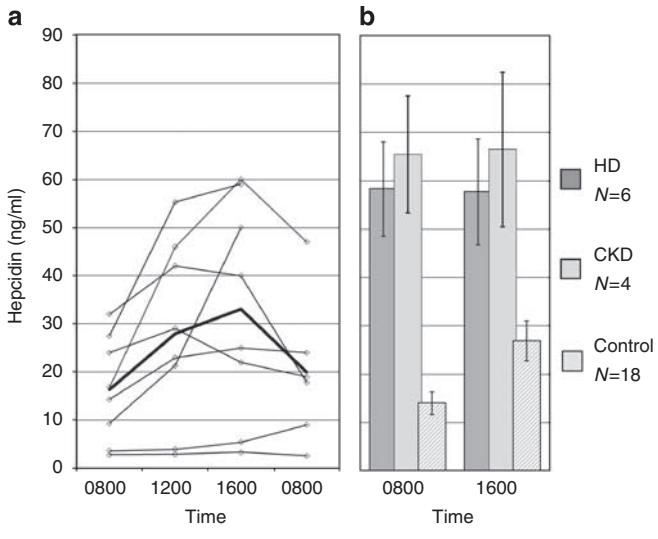

Figure 1 | Diurnal profiles of plasma hepcidin. (**a**) Diurnal profile of plasma hepcidin in eight healthy controls, with group mean (bold line). (**b**) Morning and afternoon comparisons in a further 18 controls showing a substantial rise in the afternoon ($P = 0.0001$). In four chronic kidney disease (CKD) patients and six hemodialysis (HD) patients (on a non-dialysis day) this diurnal variation in hepcidin was not observed.

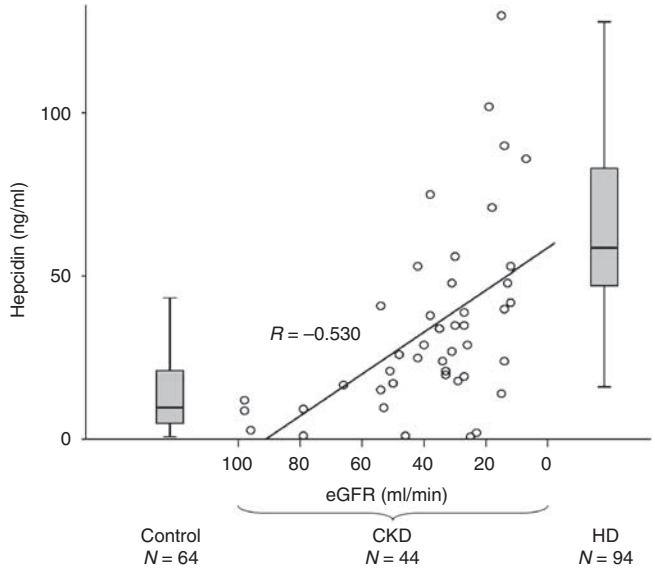

Figure 2 | Plasma hepcidin levels in healthy controls, patients with chronic kidney disease (CKD), and hemodialysis patients (HD). CKD group is shown as a scatterplot with linear regression ($P = 0.0002$). Group medians are significantly different at 10.8, 26.5, and 58.5 ng/ml ($P < 0.001$ for control vs CKD, and $P < 0.0001$ for CKD vs HD).

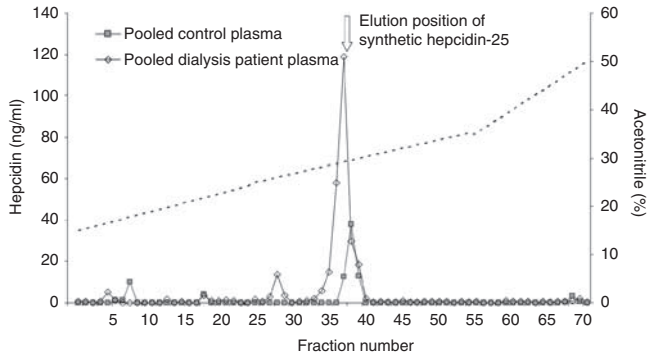

Figure 3 | Elution profile of hepcidin immunoreactivity following high-performance liquid chromatography separation of pooled control and hemodialysis plasma. As expected, a single peak due to hepcidin-25 is present in control plasma. In hemodialysis plasma a small second peak is also present, but over 90% of the immunoreactivity is chromatographically identical to hepcidin-25.

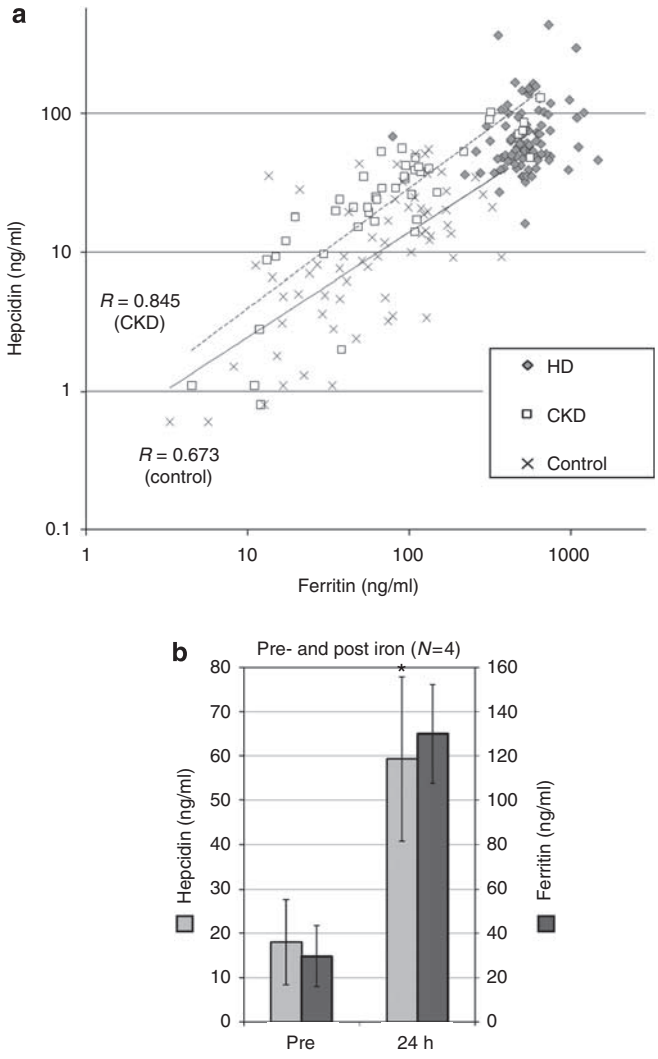

Figure 4 | Relationship between iron status and plasma hepcidin. (**a**) Correlation between log hepcidin and log ferritin in healthy controls (solid line, $R = 0.673$, $P < 0.0001$), patients with chronic kidney disease (CKD, dotted line, $R = 0.845$, $P < 0.0001$ for univariate, $\beta = 0.745$, $P < 0.001$ for model including eGFR), and hemodialysis patients (HD). The correlation is lost in the HD group due to high ferritin levels associated with intravenous iron treatment. The relationship between ferritin and hepcidin is similar in CKD and control groups. (**b**) In CKD patients receiving intravenous iron for the first time, hepcidin rises promptly after infusion of 200 mg iron sucrose ($P = 0.047$). All samples were taken at 1500 hours.

58.3 vs 57.8 ng/ml in HD group). Pre- and post-dialysis samples were also taken from six patients, showing no reduction following a standard dialysis session (68.7 vs 69.7 ng/ml for pre- and post-dialysis, respectively).

The relationship between hepcidin and ferritin was preserved in CKD patients ($R = 0.845$, $P < 0.0001$; Figure 4a) and by multivariate regression, ferritin was a stronger predictor than eGFR, although significant correlations remained for eGFR after correction for ferritin ($\beta = -0.230$, $P = 0.017$) as well as for ferritin after correction for eGFR ($\beta = 0.745$, $P < 0.001$). In the HD group, this relationship with ferritin was no longer observed, possibly because there was less variation in levels, which were high due to target-driven treatment with intravenous iron (Figure 4a). To confirm that hepcidin responds to iron status despite renal failure, samples were taken from four iron-naive CKD patients before and after administration of a first dose of intravenous iron. Hepcidin levels in these patients increased from 18.1 ± 9.6 to 59.3 ± 18.6 ng/ml at 24 h after infusion ($P = 0.047$; Figure 4b).

To examine whether elevated plasma hepcidin may be partly attributable to inflammatory signals, we measured C-reactive protein and IL-6, and identified no correlation with hepcidin in CKD ($R = 0.20$, $P = 0.19$ for C-reactive protein and $R = 0.28$, $P = 0.07$ for IL-6) or HD patients ($R = 0.04$, $P = 0.69$ for C-reactive protein and $R = 0.12$, $P = 0.26$ for IL-6).

It has been hypothesized that elevated hepcidin may explain reduced sensitivity to EPO in dialysis patients.[26,27] To investigate this, we compared hepcidin level, hemoglobin, and EPO dose in dialysis patients (Figure 5a). High hepcidin levels were associated, as expected, with low hemoglobin ($\beta = -0.290$, $P = 0.004$), but contrary to the hypothesis, were also strongly and independently related to low EPO dose ($\beta = -0.335$, $P = 0.001$), regardless of whether EPO dose was measured as an absolute value, or normalized by body weight or by hemoglobin. This correlation was consistent across each tertile of hemoglobin (Figure 5b). The relationship between hepcidin and other clinical parameters in this group is shown in Tables 1 and 2.

To confirm the suspicion that EPO therapy may be responsible for reducing hepcidin, we measured levels in seven CKD patients as they were starting EPO (20 μg darbepoietin alfa weekly) for the first time (Figure 6). Plasma hepcidin fell from 70.0 ± 4.7 to 60.7 ± 6.0 ng/ml during the first few days ($P = 0.045$) and remained at similar levels when patients were retested after 2–4 weeks of continued therapy.

A potential mechanism by which hepcidin might be reduced by EPO involves growth differentiation factor 15

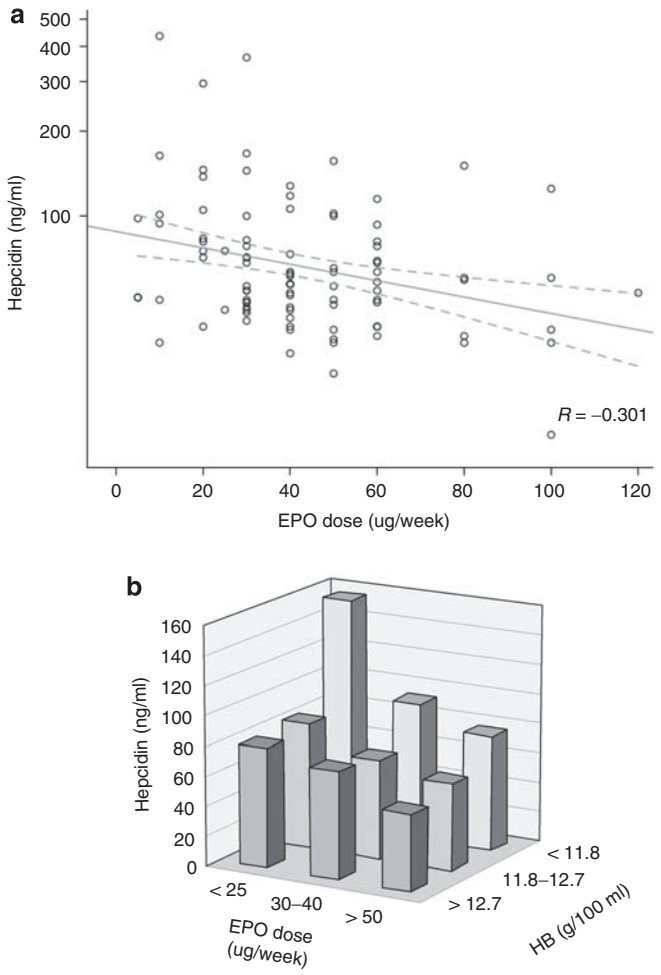

Figure 5 | Relationship between erythropoietin dose and plasma hepcidin in dialysis patients. (**a**) Inverse correlation between log hepcidin and erythropoietin (EPO) dose in dialysis patients ($R = -0.301$, $P = 0.003$ for univariate, $\beta = -0.335$, $P = 0.001$ for model including hemoglobin). Dashed lines show 90% CI for linear regression. (**b**) Plasma hepcidin by tertiles of EPO dose and hemoglobin (HB), showing that the association of higher EPO dose with lower hepcidin is conserved across hemoglobin concentrations and that, within each EPO dose tertile, lower hemoglobin is associated with higher hepcidin.

(GDF15), which may function as a signal from active bone marrow to suppress hepcidin expression.[28] We found that in the HD group, plasma GDF15 was significantly correlated with EPO dose ($R = 0.205$, $P = 0.047$), consistent with its role as a marker of marrow activity, but there was no correlation between plasma GDF15 concentrations and hepcidin levels ($R = -0.002$, $P = 0.98$). However, GDF15 levels were positively correlated with hepcidin levels in the CKD group ($R = 0.593$, $P = 0.002$). Together, these results suggest that altered hepcidin levels in renal disease are not due to changes in circulating GDF15.

DISCUSSION

These data confirm, using a novel assay, previous observations that hepcidin levels rise during the day and are elevated in renal impairment,[29,30] providing a possible mechanism for the impaired iron absorption observed in renal disease.[1] Our findings also demonstrate a number of important additional points. First, the normal diurnal variation in hepcidin is suppressed in those with reduced levels due to low iron stores, or elevated levels due to severe renal impairment.

Second, even in the presence of moderate renal impairment, hepcidin remains closely related to iron stores, and responsive to changes in iron status, without any obvious relationship to the inflammatory mediators measured.

Third, although high hepcidin is associated with low hemoglobin, consistent with its role in restricting erythropoiesis, it is also strongly and independently associated with low EPO dosage. Rather than reflecting hepcidin's influence on EPO requirement, and potential as a marker of EPO resistance, these data are consistent with a model in which hepcidin levels are directly or indirectly suppressed by EPO. This concept is supported by the reduction in hepcidin seen in CKD patients starting EPO therapy. Such an effect on hepcidin expression is also suggested by animal experiments in which stimulation of erythropoiesis by venesection or EPO administration is sufficient to suppress hepcidin mRNA levels.[8,31]

In conclusion, we have shown that a raised plasma hepcidin level, with flattening of the normal diurnal variation, occurs in kidney disease. This increase in hepcidin level reflects both the degree of renal impairment and iron storage, but is independent of variation in markers of inflammation. The results also suggest that EPO therapy can suppress hepcidin, ameliorating disordered iron transport as well as treating EPO deficiency.

Table 1 | Continuous clinical parameters in the hemodialysis group

	Mean	5th percentile	95th percentile	β	*P*
Age	64.6	39.2	83.0	−0.082	0.414
Ferritin* (ng/ml)	550	291	1014	0.247	0.013
Body mass index (kg/m^2)	25.2	19.3	32.1	−0.085	0.397
Albumin (g/l)	34.4	28.0	41.0	0.101	0.319
Cholesterol (mmol/l)	3.55	2.40	5.20	−0.236	0.018
Creatinine (μmol/l)	669	405	886	0.133	0.178
spKt/V	1.81	1.47	2.13	0.051	0.632
C-reactive protein* (mg/l)	12.9	2.8	31.2	−0.016	0.874
Interleukin-6* (pg/ml)	5.2	1.2	12.8	0.195	0.054
GDF15 (ng/ml)	4.25	2.27	7.28	0.072	0.467

spKt/V, hemodialysis dose; GDF15, growth differentiation factor 15.
Distributions of clinical parameters in the hemodialysis group, and correlation with hepcidin in a model adjusted for erythropoietin and hemoglobin. (*skewed variables that were normalized by log transformation for regression analysis).

Table 2 | Gender and comorbidity in the hemodialysis group

	%	Hepcidin ratio	*P*
Sex (male)	66.0	0.74	0.079
Residual renal function	37.9	1.09	0.739
Diabetes	36.6	1.16	0.375
Vascular disease	52.1	1.23	0.217

Ratio of plasma hepcidin levels measured in men vs women, and in the presence vs absence of significant residual renal function and comorbid conditions.

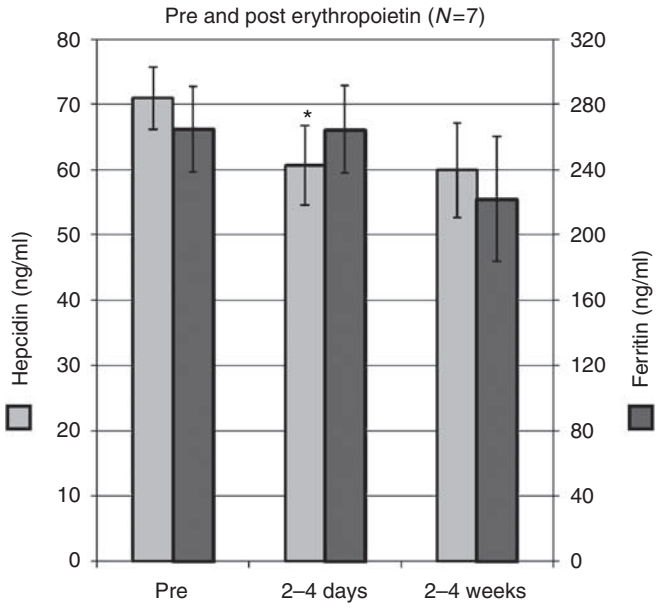

Figure 6 | Effect of erythropoietin on hepcidin levels. On commencing erythropoietin treatment in chronic kidney disease patients, hepcidin falls ($P = 0.045$ at 2-4 days, $N = 7$) remaining at the lower level on continued therapy ($N = 5$). All samples were taken at 1500 hours.

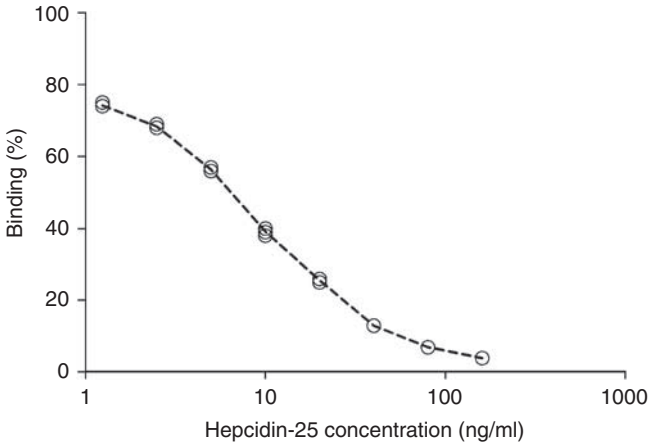

Figure 7 | Hepcidin radioimmunoassay standard curves. Assays ($n = 5$) performed in serum-free buffer over the range 1.25–160 ng/ml, on 5 days, with mean curve shown.

MATERIALS AND METHODS

This study was approved by the local NHS Research Ethics Committee (06/Q0406/134) and all participants gave informed consent according to the Declaration of Helsinki principles. Renal patients were recruited from a regional center with mixed ethnicity, mainly Caucasian and South Asian. All samples were taken into heparinized tubes with immediate centrifugation and frozen storage.

Hepcidin was measured by a novel competitive immunoassay: polyclonal rabbit anti-hepcidin-25 antibody was produced by immunization of a female New Zealand white rabbit with synthetic hepcidin-25 (Bachem, UK) conjugated to Keyhole limpet hemocyanin. Binding was assessed by competition with I^{125}-labeled hepcidin-25, separated by secondary antibody (mean binding standard curve is shown in Figure 7). The assay is linear up to 200 ng/ml, with a detection limit of 0.6 ng/ml, intraassay precision 7.2% (3 ng/ml) and 5.8% (35 ng/ml), and interassay precision 7.6% (3 ng/ml) and 6.7% (35 ng/ml). Analytical recovery was 98% (5 ng/ml) and 97% (40 ng/ml). No cross-reactivity with human insulin, glucagon, angiotensinogen, β-defensin 1–4 or α-defensin 1 proteins was observed. As expected, some cross-reactivity with synthetic hepcidin-20 (Peptide Institute, Osaka, Japan) was observed, but was only 9.6% (200 ng/ml). There was no cross-reactivity with prohepcidin, as demonstrated by measurement of hepcidin and prohepcidin immunoreactivity (enzyme-linked assay from DRG International, NJ, USA) in HPLC-fractionated control plasma (Figure S1).

High performance liquid chromatography (HPLC) was performed on pooled control ($n = 5$) and HD ($n = 5$) plasma samples, preextracted using C^{18} Sep-Pak Cartridge (recovery $>80\%$). Extracted HPLC analyzed 1 ml/min collected fractions using Discovery BIO Wide Pore C^{18} Column with a 15:45% ACN:H_2O gradient for 70 min. Eluted fractions were then assayed using hepcidin RIA for immunoreactive fragments.

IL-6 and GDF15 were measured by enzyme-linked assays (R&D Systems, Abingdon, UK). Differences between groups were measured using Student's *t*-test, and associations between parameters were measured using Pearson's correlation coefficient. Skewed variables were log-transformed. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for multivariate regression analysis using variables significant in bivariate correlations.

DISCLOSURE

PHM is a scientific founder, director and holds equity in ReOx Ltd.

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SUPPLEMENTARY MATERIAL

Figure S1. Elution profile of hepcidin and prohepcidin immunoreactivity following high-performance liquid chromatography fractionation of pooled control plasma ($n = 5$), preextracted using C^{18} Sep-Pak Cartridge (recovery $>80\%$).

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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