HFE Mutations Modulate the Effect of Iron on Serum Hepcidin-25 in Chronic Hemodialysis Patients

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Background and objectives: Increased serum hepcidin has been reported in patients receiving chronic hemodialysis, and hypothesized to contribute to the alterations of iron metabolism of end-stage renal disease. However, no quantitative assessment is available to date; the clinical determinants are still under definition; and the role of genetic factors, namely HFE mutations, has not yet been evaluated. The aim of this study was to quantitatively assess serum hepcidin-25 in hemodialysis patients *versus* controls, and analyze the relationship between hepcidin, iron indices, HFE genotype, and erythropoietic parameters.

Design, setting, participants & measurements: Sixty-five hemodialysis patients and 57 healthy controls were considered. Hepcidin-25 was evaluated by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, HFE genotype by restriction analysis.

Results: Serum hepcidin-25 was higher in hemodialysis patients compared with controls. In patients, hepcidin-25 correlated positively with ferritin and C reactive protein, and negatively with serum iron after adjustment for confounders. Hepcidin/ferritin ratio was lower in patients with (n = 25) than in those without (n = 40) HFE mutations. At multivariate analysis, hepcidin-25 was independently associated with ferritin and HFE status. In a subgroup of 22 "stable" patients, *i.e.*, with Hb levels on target, normal CRP levels, and absence of complications for at least 1 yr, hepcidin-25 was negatively correlated with Hb levels independently of confounders.

Conclusions: Serum hepcidin-25 is increased in hemodialysis patients, regulated by iron stores and inflammation, and relatively reduced in subjects carrying frequent HFE mutations. Hepcidin-25 may contribute to the pathogenesis of anemia by decreasing iron availability.

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Increased serum levels of hepcidin, the novel iron hormone inhibiting iron absorption from enterocytes and iron recycling from macrophages, have recently been reported in patients with end-stage renal disease (ESRD), and hypothesized to contribute to the alterations of iron metabolism and anemia of ESRD (1–5).

Anemia related to renal failure, blood losses, and chronic inflammation is an almost universal finding in patients with ESRD receiving chronic hemodialysis treatment (CHD), and is associated with increased mortality (6,7). Treatment is based on erythropoiesis stimulating agents (ESAs) in association with i.v. iron formulations (8). Functional iron deficiency is a common finding, determining the need for high doses of ESAs and iron, both associated with adverse events. High doses of ESAs increase the risk of mortality due to cardiovascular events related to hypertension and hypercoagulability (9–11), whereas excess iron promotes vascular damage by inducing oxidative stress and heightens the risk of infections (12–16). The mechanism proposed to explain refractoriness to i.v. iron is related to the inhibition of erythropoiesis and iron recycling from macrophages by inflammation (17).

The alteration of iron metabolism of CHD is characterized by decreased transferrin saturation, resulting in reduced iron availability for erythropoiesis, and is supposed to be related to chronic inflammation and/or blood losses (18). Hyperferritinemia is also frequently observed, and although in the most severe cases excess iron administration is believed to play a role, in the majority of cases it is thought to reflect the malnutrition inflammation cachexia syndrome (19). It has been shown that inflammation and oxidative stress induce iron retention in monocytes/macrophages and the transcription of ferritin, a protein with anti-oxidant activity (20,21). However, recent data

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highlight that ferritin levels reflect also iron stores in CHD patients. Indeed, in these subjects ferritin correlated positively with transferrin saturation, with the presence of common mutations of the HFE gene of hereditary hemochromatosis (12), and with bone marrow iron stores (22).

HFE mutations, the most common cause of iron overload in Caucasians (23), disrupt the physiologic regulation of hepcidin by hepatic iron stores. Hepcidin inhibits intestinal iron absorption and iron recycling from monocytes by binding and inactivating the iron exporter Ferroportin-1 (Fp-1) (24). Furthermore, hepcidin is an acute phase reactant induced by inflammation and is downregulated by anemia, hypoxia and erythropoietin (5,25,26).

Until very recently, no reliable and practical assay to measure serum hepcidin was available. Thus, the effect of ESRD and CHD on the regulation of hepcidin, which is physiologically excreted with urine, and the influence of hepcidin on the regulation of iron metabolism in patients with ESRD are still under definition. In previous studies, elevated levels of serum pro-hepcidin, an immature form of hepcidin measured by ELISA, were found in patients with ESRD (2). However, pro-hepcidin levels have not been found to correlate with mature biologically active hepcidin and with iron stores (1,27).

An increase in serum hepcidin-25, the biologically active form of the hormone, measured by a semiquantitative approach, has recently been reported in studies including small samples of CHD patients. Interestingly, hepcidin levels were correlated with ferritin in these subjects (1,4). Theoretically, increased hepcidin levels may reduce intestinal iron absorption and iron recycling from monocytes (24), decreasing serum iron available for the erythropoiesis (28) and the therapeutic effect of ESAs and i.v. iron, as commonly observed in CHD patients (3). Supporting this hypothesis, we recently reported that frequent mutations in the HFE gene, which decrease hepcidin response to iron stores (29,30), are associated with increased sensitivity to ESAs and iron in CHD patients, and may be associated with a better clinical outcome (31). However, in a study considering a series of 24 patients, hepcidin-25 was not significantly associated with r-HuEpo responsiveness (1).

The aim of this study was to evaluate serum hepcidin-25 levels by a quantitative approach (27) in CHD patients treated with a standardized protocol according to current guidelines and in controls, and to evaluate in CHD the possible relationship of hepcidin with markers of iron status, the HFE genotype and erythropoietic parameters.

Materials and Methods

Sixty-five of 110 prevalent CHD patients treated at the Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena IRCCS of Milan, with available serum and DNA samples, were included. Exclusion criteria were active major infectious diseases (pneumonia, sepsis), neoplasia, congestive heart failure (NYHA stage III and IV), unstable ischemic heart disease, hepatic failure, and the β-thalassemic trait. Part of this series has previously been described elsewhere in detail (12,31).

Twenty-two of these patients could be considered "stable", *i.e.,* had Hb levels on target (between 10.5 and 12 g/dl during the last 12 mo before enrollment, without changes in the dose of recombinant human erythropoietin [r-HuEpo] and iron), normal CRP levels (<4 mg/dl) and absence of complications (clinically detectable bleeding and hospitalization) for at least one year before enrollment. These stable patients were characterized by younger age ($P = 0.02$), higher ferritin levels ($P = 0.001$) and lower CRP levels ($P = 0.0002$), compared with the others.

Patients were dialyzed with synthetic biocompatible membranes and bicarbonate dialysate, and given i.v. r-HuEpo (Eprex®) t.i.w. at a dose aimed to maintain hemoglobin (Hb) between 10.5 and 12 g/dl. Iron was administered i.v. as Fe^{3+}-gluconate (Ferlixit®) when transferrin saturation was <30% or ferritin <200 ng/ml, and suspended when ferritin was above 500 ng/ml, as suggested by guidelines (8). Iron infusion was started b.i.w. and titrated according to requirements. Clinicians were unaware of *HFE* genotypes and hepcidin dosage of the patients throughout the duration of the study.

Blood samples for complete blood count, iron parameters (tested by standard methods) and hepcidin-25 were collected before hemodialysis at standardized times after the last administration of therapies, potentially altering iron status and hepcidin release: 1 wk after the last dose of i.v. iron, and 3 d after the last dose of r-HuEpo in all patients. At the time of evaluation, all patients were receiving a defined amount of iron and r-HuEpo to support erythropoiesis.

HFE genotype was determined by restriction analysis (32).

Fifty-seven gender-matched controls were enrolled among healthy volunteers participating in a phase II trial at the Centre for Clinical Research of the Azienda Ospedaliera-Universitaria di Verona, as described previously in detail elsewhere (33). Briefly, at enrollment they completed a questionnaire with specific items relevant to iron metabolism (*i.e.,* any history of blood donations, previous pregnancy, menstrual losses, etc.) and were evaluated by laboratory studies including complete blood count, serum iron, transferrin saturation, ferritin, inflammatory markers (including CRP), liver function tests and creatinine. To be considered as appropriate "normal controls" for the serum hepcidin assay, all these parameters were required to be normal.

Clinical features of subjects included in the study are shown in Table 1.

Each patient gave written informed consent. The study was conducted according to the principles contained in the Declaration of Helsinki. The protocol was approved by the Institutional Review Boards of the Ospedale Maggiore Policlinico Mangiagalli Regina Elena Fondazione IRCSS of Milano, and of the Azienda Ospedaliera-Universitaria of Verona.

Serum Hepcidin Assay

We used a protocol based on PBSCIIc mass spectrometer and copper-loaded immobilized metal-affinity capture ProteinChip arrays (IMAC30-Cu^{2+}). Five μl of serum were applied to an IMAC30-Cu^{2+} surface that binds hepcidin based on its affinity for Cu^{2+} ions. The binding surface was equilibrated and washed with appropriate buffers according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Subsequent work-up, SELDI-TOF MS instrumental settings, read out, and data analysis are described elsewhere (33), with the addition that protein chip handling was performed in a nitrogen atmosphere to prevent methionine oxidation (27). A synthetic hepcidin analogue (hepcidin-24, Peptides International, Louisville, KY) was used as an internal standard (27). Concentrations of serum hepcidin-25 were expressed as nM. Hepcidin-25 concentration of 0.5 nM (half the lower detectable concentration in these series) was arbitrarily assigned to samples with undetectable serum hepcidin-25. The intra- and interassay coefficient of variations of this method ranged from 6.1 to 7.3 percent, and from 5.7 to 11.7 (mean 7.7) percent, respectively (29).

Table 1. Clinical features of 57 healthy controls and 65 Italian CHD patients

	Controls $n = 57$	Overall Series of Patients $n = 65$	Stable Patients $n = 22$
Age years	35 ± 15	65.5 ± 12	61.9 ± 9
Sex F	21 (36.8)	26 (40)	9 (40.9)
Dialysis duration months	-	35.5 {19-87}	56 {24-112}
Hypertension	-	22 (34)	10 (45)
Diabetes	0	8 (12.3)	1 (4.5)
Smoke	-	19 (29)	7 (32)
Albumin g/dl	-	3.7 ± 0.5	3.6 ± 0.5
PTH	-	360 {137-780}	365 {115-611}
Hb g/dl	14.4 ± 1.2	10.8 ± 1.1	10.9 ± 0.7
r-HuEpo IU/Kg/wk	0	90.9 {36-137}	87.3 {42-142}
Iron mg/mo	0	67.5 {10-127}	86 {51-136}
Ferritin ng/ml	90 {47-149}	236 {128-410}	369 {244-528}
Transferrin saturation %	28.9 ± 9	26.3 ± 10	27.8 ± 8
CRP mg/dl	0.8 {0.8-1.4}	2.3 {0.8-4.3}	0.85 {0.4-2.3}
HFE genotype			
wt/wt	-	40 (61.5)	11 (50)
H63D/wt	-	18 (27.7)	9 (40.9)
C282Y/wt	-	4 (6.2)	0
H63D/H63D	-	3 (4.6)	2 (9.1)

( ), % values; { }, interquartile range; wt, wild-type.

Statistical Analysis

Since the C282Y and H63D are both loss-of-function HFE mutations, and are not associated with iron overload in the general population (23), but are both associated with more favorable erythropoietic parameters in patients with CHD (31), we grouped together, for the primary analysis, subjects positive for either one of the two mutations. We also present results for the effect of specific HFE genotypes.

Results are expressed as means ± SD for normally distributed variables and as median {interquartile range} for non-normally distributed variables, including serum hepcidin-25 levels, ferritin and CRP. Quantitative variables were correlated by Spearman's rho test, and hepcidin-25 levels were compared between groups by the Wilcoxon test. To evaluate the effect of HFE genotype on the hepcidin-25 response to iron stores, we also evaluated the hepcidin-25/ferritin ratio, an established marker of adequacy of hepcidin response to iron stores (33,34). Independent predictors of serum hepcidin-25, Hb levels, and serum iron levels were evaluated by multivariate analysis (generalized linear model) including variables identified at univariate analysis. Results were considered significant when p was <0.05 (two-tailed). Analyses were carried out with the JMP 6.0 statistical analysis software (SAS Institute Inc, Cary, NC).

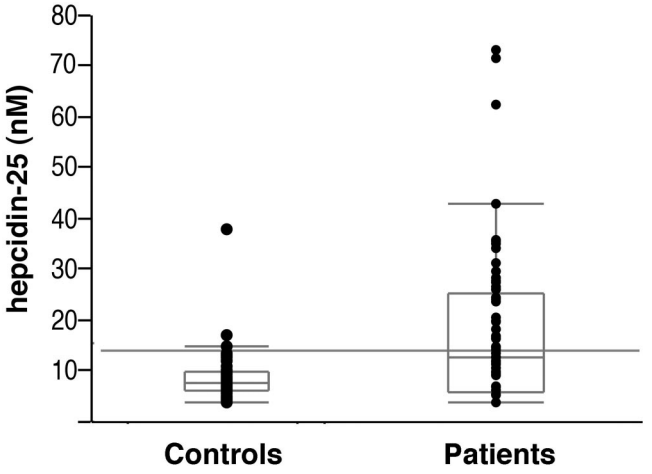

Figure 1. Hepcidin-25 levels in 57 healthy controls and 65 CHD patients ($P = 0.0021$). Outlier box plots represent interquartile ranges, whereas lines that cross the boxes indicate the median value. The whiskers extend to the 1.5x interquartile range distances.

Results

Hepcidin-25 was significantly higher in patients compared with controls (10.63 {2.67 to 23.66} *versus* 4.62 {3.01 to 7.02} nM, $P = 0.0021$) (Figure 1).

Correlations of hepcidin-25 with clinical variables including iron indices, inflammation and erythropoietic parameters in CHD patients are shown in Table 2. In the whole CHD population, hepcidin-25 was not correlated with demographic and anthropometric features, the duration of dialysis and measures of dialysis adequacy. Hepcidin-25 was significantly correlated with serum ferritin (Spearman's rho = +0.46; $P = 0.0001$, Figure 2), but not with transferrin saturation, the dose of iron and r-HuEpo. We observed a trend for a negative correlation between hepcidin-25 and serum iron (rho = −0.20; $P = 0.1$). Hepcidin-25 was also correlated with CRP levels (rho = +0.27; $P = 0.029$, Figure 2).

At multivariate analysis considering hepcidin-25, ferritin, CRP, monthly iron dosage and the presence of HFE mutations, serum iron was independently associated with hepcidin-25

Table 2. Correlation between hepcidin-25 levels and clinical features in 65 Italian patients receiving CHD

	Overall Series (n = 65)		Stable Patients (n = 22)	
	rho	p	rho	p
Age	+0.01	0.91	-0.01	0.97
Dialysis duration	+0.03	0.82	-0.07	0.76
Hemoglobin g/dl	+0.05	0.67	-0.51	0.015
r-HuEpo IU/Kg/wk	+0.002	0.99	+0.25	0.26
Serum iron μg/dl	-0.274	0.15	-0.38	0.08
Transferrin saturation %	-0.003	0.99	-0.28	0.21
Ferritin ng/ml	+0.46	0.0001	+0.42	0.05
Iron mg/month	-0.159	0.35	+0.12	0.59
CRP mg/dl	+0.27	0.029	+0.1	0.66

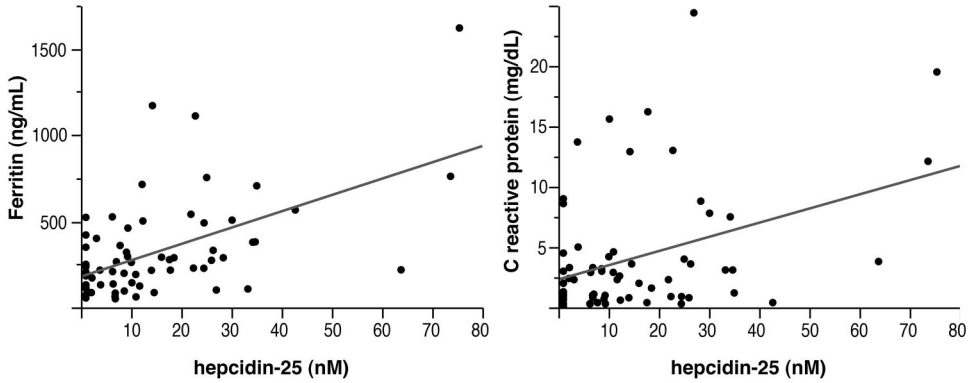

Figure 2. Correlation between hepcidin-25, ferritin and CRP in 65 Italian CHD patients (P = 0.0001 and P = 0.029, respectively, at Spearman's test).

(parameter estimate −0.81, P = 0.014), monthly iron dose (estimate −0.08, P = 0.04) and the presence of HFE mutations (estimate + 11.32, P = 0.017).

The effect of HFE genotype on hepcidin-25 levels and on the hepcidin-25/ferritin ratio (nM/ng/ml) is shown in Figure 3. Hepcidin-25/ferritin ratio was significantly lower in patients positive (n = 25, 38.5%) compared with those who were negative (n = 40, 61.5%) for HFE mutations (0.02 {0.010 to 0.062} *versus* 0.05 {0.018 to 0.095}; P = 0.045).

At multivariate analysis considering as independent variables ferritin, CRP levels, and the presence of HFE mutations, hepcidin-25 levels were independently associated with ferritin (parameter estimate + 0.03, $P < 0.0001$), and the absence of HFE mutations (estimate + 4.05, P = 0.0184), but not significantly associated with CRP (estimate + 0.56, P = 0.103).

In a subgroup of 22 "stable" patients, as defined in the Methods and Materials section (whose clinical features are shown in Table 1), hepcidin-25 was correlated with ferritin (rho = +0.42; P = 0.05) and negatively correlated with Hb levels (rho = −0.51; P = 0.015) (Table 2). We also observed a trend for a negative correlation with serum iron (rho = −0.38; P = 0.08). In this subgroup, at multivariate analysis considering hepcidin-25, sex, transferrin saturation %, ferritin, r-HuEpo dose, iron dose, and CRP, Hb was independently associated with hepcidin-25 (estimate −0.062; P = 0.0011) and CRP (estimate −0.179; P = 0.017).

Discussion

To the best of our knowledge, this is the largest series of CHD patients analyzed for serum hepcidin levels, and the first one in which a quantitative assay was used. We also investigated the effect of common HFE gene mutations on hepcidin-25, and provide data supporting the possible role of hepcidin-25 in the pathophysiology of the alterations of iron metabolism and anemia.

Our data clearly confirm and extend previous reports based on a semiquantitative assay in smaller series, suggesting substantial hepcidin-25 upregulation in CHD patients compared with controls (4,5), and preserved regulation of hepcidin-25 by iron stores and inflammation in this setting (1,4). They also provide evidence of a modulating effect of HFE mutations on hepcidin-25 regulation by iron stores.

First, we showed that hepcidin-25 levels are higher in patients receiving CHD than in healthy controls. Notably, while the groups studied were matched for gender, which is a major determinant of serum hepcidin levels (27,35), CHD patients were significantly older than controls. However, this is unlikely to represent a substantial bias, since previous studies did not

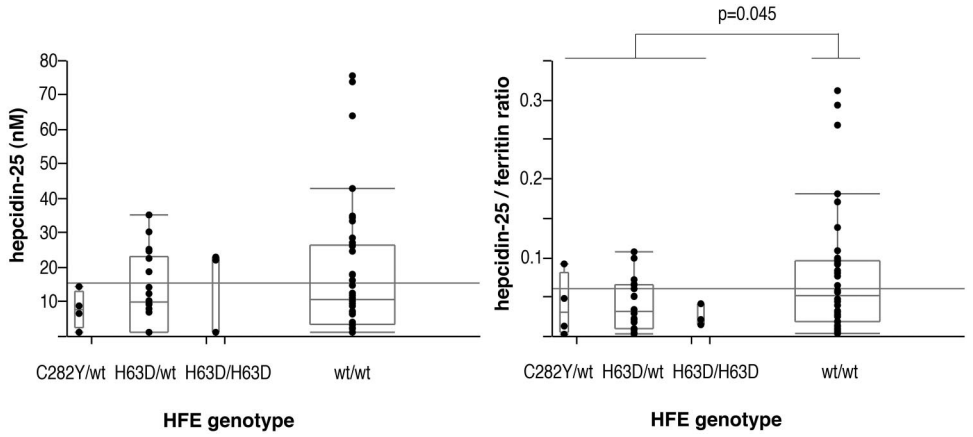

Figure 3. Hepcidin-25 quantitative levels (nM) and hepcidin-25/ferritin ratio (nM/ng/ml) in 65 CHD patients subdivided according to HFE genotype. Outlier box plots represent interquartile ranges, whereas lines that cross the boxes indicate the median value. The whiskers extend to the 1.5x interquartile range distances.

show a consistent increase in hepcidin levels with age in the general population (35). These data suggest that ESRD itself plays a role in hepcidin-25 accumulation in this setting. Whether different dialytic techniques may affect the rate of hepcidin-25 clearance in CHD, and the consequences on iron metabolism and erythropoiesis, must be ascertained by future studies.

Also, we showed preserved regulation of hepcidin-25 levels by iron stores, as demonstrated by the very strong correlation with serum ferritin, and modulation by inflammation, as detected by CRP levels. Again, these data match closely those previously obtained, showing a correlation of hepcidin-25 with ferritin and IL-6, but not CRP, in CHD, and, anecdotally, an increase in hepcidin-25 after i.v. iron administration (1,4). Moreover, for the first time we were able to demonstrate a negative correlation between hepcidin-25 and serum iron. These results imply that in CHD, excessive iron administration may paradoxically hamper iron utilization for erythropoiesis by trapping iron in phagocytes, because of excessive hepcidin-25 induction favored by chronic inflammation, and that the effect of inflammation on altered iron metabolism and erythropoiesis may be mediated by increased hepcidin levels. They also suggest that pharmacologic downregulation of hepcidin (36) may be beneficial in CHD.

This hypothesis is supported by recent data by our group indicating that frequent HFE mutations, affecting about 30% of Caucasians and believed to cause a relative deficit in hepcidin release (30,34), are associated with higher iron availability, more efficient erythropoiesis, and possibly a better prognosis (31). In the general population, the C282Y HFE mutation is detected in about 3 to 10%, whereas the H63D in about 22 to 28% of subjects. However, only the C282Y +/+, C282Y +/− H63D +/− and H63D +/+ HFE genotypes have been associated with increased susceptibility to develop iron overload and clinical manifestations of hemochromatosis (23). In subjects without ESRD, chronic inflammation and liver disease, simple heterozygosity for the H63D or the C282Y HFE mutations was not found to be associated with either iron overload (23) or reduced hepcidin release (33), and previous data obtained in a very limited number of CHD patients did not support an influence of increased hepcidin on r-HuEpo requirement (1).

To clarify this important issue, we analyzed the effect of HFE mutations on hepcidin-25 levels and found that the presence of either the H63D or the C282Y mutations was associated with reduced induction of hepcidin-25 by iron stores, and was an independent predictor of hepcidin-25 levels at multivariate analysis. These results clearly suggest that in CHD, simple heterozygosity for HFE mutations alters iron metabolism by decreasing hepcidin levels. The subtle effect of these molecular variants is likely magnified in CHD patients by the environmental pressure determined by chronic inflammation and exposure to high amounts of iron and ESAs.

Furthermore, we found that in a subgroup of patients with stable disease, selected to avoid the confounding effect of the frequent presence of acute inflammation, blood losses, cancer and recent variation in the dosage of therapy, hepcidin-25 negatively correlated with Hb levels and there was a trend toward a negative correlation with lower serum iron.

Since anemia and hyposideremia should rather decrease hepcidin levels, these findings suggest that hepcidin-25 plays a causal role in determining anemia by reducing iron availability to the erythron. To our knowledge, this is the first evidence supporting the theory that hepcidin is involved in the pathogenesis of the anemia of CHD.

However, results should be confirmed in larger series including patients from different centers and ethnic background, with prospective evaluation of the effect of serum hepcidin levels.

Given the possible relevance of HFE genotype for determining the prognosis and guiding therapy in patients receiving CHD, and the theoretical possibility to improve anemia management by pharmacologic modulation of hepcidin release, additional studies including larger series of patients analyzing the effect of HFE genotype on iron metabolism, erythropoiesis and clinical outcomes are required.

In conclusion, serum hepcidin-25 is increased in CHD, regulated by iron stores and inflammation, and relatively reduced

in subjects carrying frequent HFE mutations. Since increased hepcidin-25 may contribute to the pathogenesis of anemia by decreasing iron availability, the mechanisms underlying hepcidin-25 accumulation in ESRD and the therapeutic implications of these findings warrant further investigations.

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Disclosures

None.

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