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## Original Paper

# Regulation of Hepcidin-25 by Short- and Long-Acting rhEPO May Be Dependent on Ferritin and Predict the Response to rhEPO in Hemodialysis Patients

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## Key Words

Anemia · Erythropoietin · Ferritin · Hemodialysis · Hepcidin · Iron deficiency

## Abstract

**Background/Aims:** We examined whether regulation of hepcidin-25 by short- or long-acting recombinant human erythropoietin (rhEPO) is dependent on ferritin and predicts the response to rhEPO in hemodialysis (HD) patients. **Methods:** Two studies with rhEPO were performed in 9 HD patients with a 2-year interval. Serum hepcidin-25 was measured at 0–18 h after intravenous epoetin- $\beta$  (EPO) or methoxy polyethylene glycol-epoetin- $\beta$  (PEG-EPO) administration and on days 3–7 after PEG-EPO. Hemoglobin (Hb), serum ferritin, transferrin, C-reactive protein (CRP), and interleukin (IL)-6 were analyzed before hepcidin measurement and 6 months after rhEPO. Based on the serum ferritin levels before hepcidin measurement, the patients in the two studies with EPO or PEG-EPO were combined into low (11; serum ferritin of <15.0 ng/ml) and high ferritin groups (7; serum ferritin of  $\geq$ 15.0 ng/ml). The response of hepcidin-25 to rhEPO and the effect of rhEPO on anemia were compared between the groups. **Results:** The serum hepcidin-25 levels rose at 6–9 h and returned to the baseline at 18 h after EPO. They rose at 6–9 h, returned to the baseline at 18 h, and decreased on day 5–7 after PEG-EPO. Serum hepcidin-25 levels were low (<5.0 ng/ml) in the low ferritin group, but rose at 6–9 h after rhEPO in the high ferritin group. Serum transferrin levels were similar, and CRP and IL-6 were normal in both groups. Hb tended to increase in the low ferritin group, but it significantly decreased in the high ferritin group after rhEPO. **Conclusion:** Regulation of hepcidin-25 by rhEPO may be dependent on ferritin, affecting the response to rhEPO in HD patients.

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## Introduction

Iron deficiency anemia is an important problem commonly associated with patients on maintenance hemodialysis (HD). Hepcidin-25, the major form of mature hepcidin, plays an important role in the regulation of iron metabolism [1, 2]. Binding of hepcidin to its receptor ferroportin (FPN) is the principal cellular process for iron efflux, which, in turn, causes the rapid internalization and degradation of FPN [1, 2]. In duodenal enterocytes, high hepcidin prevents the movement of dietary iron into the circulation through its binding to FPN. In hepatocytes or macrophages, hepcidin prevents the efflux of stored iron into the circulation. Conversely, low hepcidin increases intestinal iron absorption and iron efflux from macrophages or hepatocytes [1, 2].

Hepcidin-25 is synthesized in the liver in response to iron overload or inflammation [1, 2]. Excess hepcidin decreases iron availability for erythropoiesis, leading to its inhibition. As described in our previous study [3], serum levels of hepcidin and ferritin have been usually increased in HD patients [4–6], and hepcidin levels were correlated positively with ferritin and negatively with erythrocyte count, hemoglobin (Hb) and hematocrit [3, 4, 6]. Similarly, serum levels of prohepcidin, a prohormone of hepcidin, were correlated negatively with Hb in HD patients [7]. In iron depletion, reflected by low serum ferritin, serum levels of hepcidin-25 were decreased, facilitating iron availability for erythropoiesis in HD patients [8]. Intravenous iron administration has been shown to increase serum levels of hepcidin in HD patients [9]. These data suggest that hepcidin levels are regulated by the iron storage status.

The long-term administration of recombinant human erythropoietin (rhEPO) affects serum levels of hepcidin or prohepcidin in HD patients [5, 10–13]. Regarding the early effect of rhEPO on hepcidin, there are a few studies using healthy volunteers showing an early and significant rise [14, 15] followed by suppression of hepcidin [14–16] after intravenous or subcutaneous administration of rhEPO. So far, there is no study on the early effect of rhEPO administration on serum hepcidin-25 in HD patients. In addition, it is unknown whether the regulation of hepcidin-25 by short- and long-acting rhEPO is different, and whether rhEPO-induced regulation of hepcidin-25 is dependent on ferritin in HD patients. Furthermore, it remains inconclusive whether an alteration of serum hepcidin-25 is predictive of the response to rhEPO in HD patients [5, 10–13]. The present study was undertaken to address these issues in HD patients.

## Subjects and Methods

The Institutional Review Board at our institution approved this study. Informed consent was obtained from all patients. Nine patients on maintenance HD without diabetes mellitus, hepatic disease, or clinical infection were enrolled in the study. To minimize intraindividual variability of serum hepcidin [17, 18] and the effect of dialysis on hepcidin [11], two independent studies for the response of hepcidin-25 to the short-acting epoetin- $\beta$  (EPO) and the long-acting methoxy polyethylene glycol-epoetin- $\beta$  (PEG-EPO) were performed using the same patients and dialysis condition with a 2-year interval.

To examine the early response of hepcidin-25 to the short- or long-acting rhEPO, serum levels of hepcidin-25 were measured at 0, 3, 6, 9 and 18 h after intravenous administration of EPO (3,000 U) or PEG-EPO (100 U). In addition, they were measured on days 3, 5, and 7 after administration of PEG-EPO. During this study, PEG-EPO was withheld for 7 days until the measurement of hepcidin-25 was completed. In two independent studies with EPO or PEG-EPO, all patients received the constant dose of the same agent of rhEPO for 6 months before, during, and 6 months after the measurement of hepcidin. As rhEPO therapy, EPO

**Table 1.** Clinical parameters before the measurement of hepcidin-25 in response to the short- or long-acting rhEPO in HD patients

Variable	Before EPO (n = 9)	Before PEG-EPO (n = 9)
Hb, g/dl	10.3±1.4	11.1±2.7
Ferritin, ng/ml	38.2±50.7	15.3±11.1
Transferrin, mg/dl	254.8±47.7	265.7±36.8
CRP, mg/ml	0.05±0.07	0.1±0.17
IL-6, pg/ml	2.3±1.2	4.3±3.1

(750–3,000 U) and PEG-EPO (100–150 U) were intravenously given 3 times a week and once a month, respectively.

Based on the baseline levels of serum ferritin before the measurement of hepcidin, the patients were divided into low (serum ferritin of <15.0 ng/ml) [19] and high ferritin groups (serum ferritin of ≥15.0 ng/ml). The patients with low or high levels of serum ferritin in two independent studies were combined and grouped into low or high ferritin groups, respectively. An alteration of serum hepcidin-25 after intravenous administration of rhEPO was compared between the groups. To determine whether an alteration of serum hepcidin-25 and ferritin is predictive of the effect of rhEPO therapy on anemia, Hb and iron parameters, including serum ferritin and transferrin, before and 6 months after the measurement of hepcidin were compared between the groups. In addition, to examine the influence of inflammation on serum hepcidin-25, serum levels of interleukin (IL)-6 and C-reactive protein (CRP) were compared between the groups. All parameters analysed were measured just before starting HD. In our series, ultrapure dialysate has been used for HD to minimize microinflammation as previously described [6].

Serum levels of hepcidin-25 were measured using a liquid chromatography-tandem mass spectrometric method as previously described [20]. Hb, serum ferritin, transferrin, and CRP were measured by the standard automated laboratory methods, and serum IL-6 by an enzyme-linked immunosorbent assay.

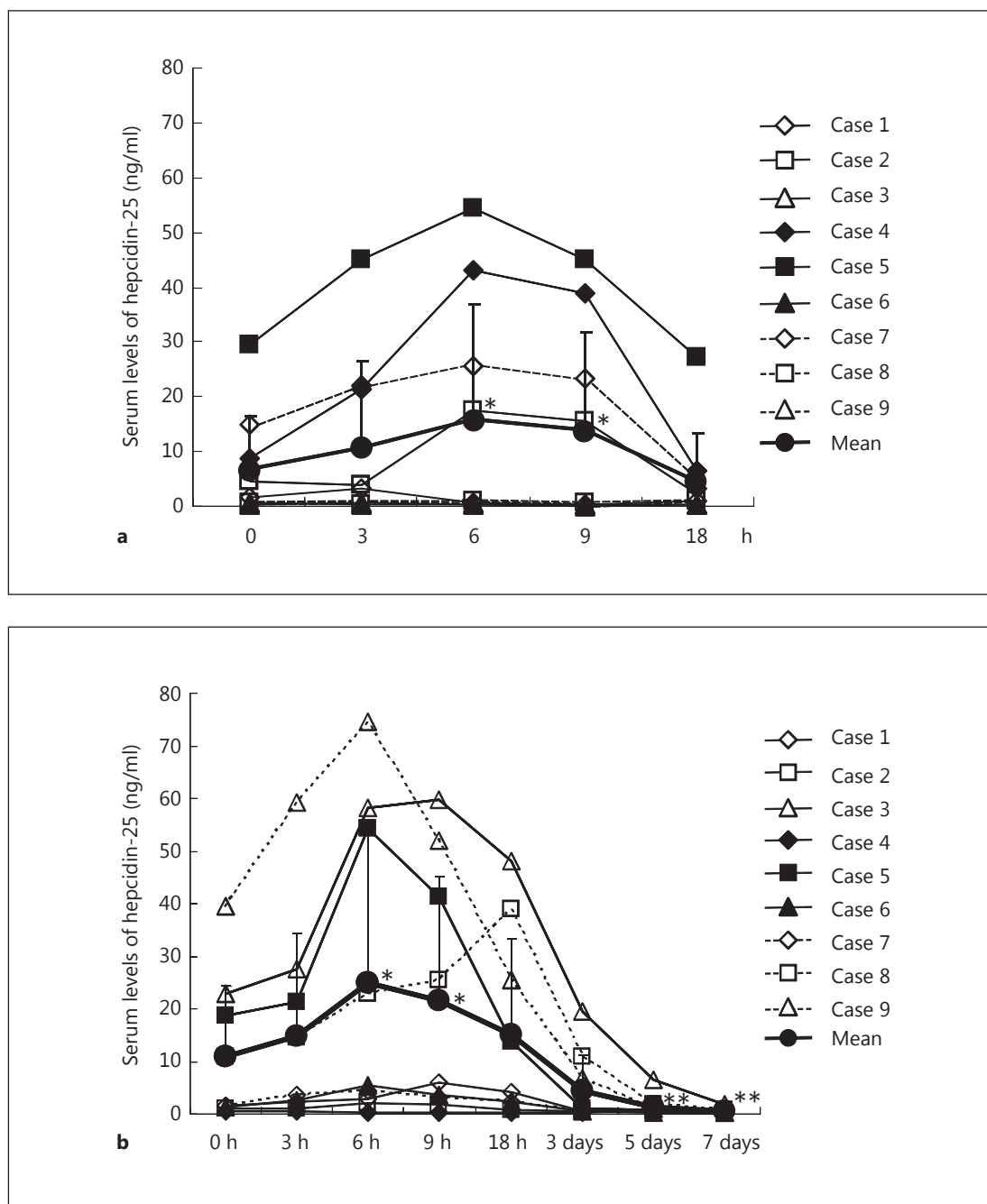
#### Statistical Analysis

Continuous data are expressed as mean ± SD and categorical data as percentage of the total population. Comparisons of two means were performed using the Mann-Whitney U test, and those of two proportions using the Fisher exact test. A software package (SPSS, version 20.0; SPSS Inc., Chicago, Ill., USA) was used for statistical analysis. A p value <0.05 was considered significant.

## Results

In 9 patients (4 males) on maintenance HD, mean age at the time of the study and duration of HD were 59.0 ± 7.0 years and 120.0 ± 95.0 months, respectively. Table 1 shows the clinical parameters of the HD patients before the measurement of hepcidin after administration of rhEPO in two independent studies. There was no significant difference in Hb, serum levels of ferritin, transferrin, CRP, and IL-6 values before the measurement of hepcidin between the studies.

Serum levels of hepcidin-25 rose from the baseline of 6.8 ± 9.8 to 15.9 ± 20.9 ng/ml (p < 0.05) at 6 h, then declined to 13.8 ± 18.0 ng/ml (p < 0.05) at 9 h, and returned to the baseline at 18 h (4.6 ± 8.7 ng/ml) after EPO administration (fig. 1a). Similarly, serum levels of hepcidin-25 rose from the baseline of 10.9 ± 13.6 to 25.0 ± 29.3 ng/ml (p < 0.01) at 6 h, then



**Fig. 1.** Alteration of serum levels of hepcidin-25 in response to the intravenous administration of EPO (**a**) and PEG-EPO (**b**) in patients on maintenance HD. **a** \*  $p < 0.05$ , 0 vs. 6 and 9 h. **b** \*  $p < 0.01$ , 0 vs. 6 and 9 h, \*\*  $p < 0.05$ , 0 h vs. day 5 and day 7.

declined to  $21.5 \pm 23.8$  ng/ml ( $p < 0.01$ ) at 9 h, and returned to the baseline at 18 h ( $15.2 \pm 18.1$  ng/ml) after PEG-EPO administration (fig. 1b). Serum levels of hepcidin-25 on day 3 ( $4.5 \pm 6.7$  ng/ml) tended to be decreased, but did not differ significantly from the baseline. However, they were significantly decreased on day 5 ( $1.4 \pm 1.9$  ng/ml,  $p < 0.05$ ) and day 7 ( $0.5 \pm 0.4$  ng/ml,  $p < 0.05$ ) after PEG-EPO administration, compared to the baseline.

**Table 2.** Dose of rhEPO and serum levels of ferritin before the measurement of hepcidin-25

Case	Study with EPO		Study with PEG-EPO	
	EPO dose, U/week	Serum ferritin before the measurement of hepcidin-25, ng/ml	PEG-EPO dose, U/month	Serum ferritin before the measurement of hepcidin-25, ng/ml
1	2,250	12.1*	100	13.2*
2	2,250	19.3	100	11.8*
3	9,000	6.1*	150	30.8
4	4,500	33.1	100	6.5*
5	4,500	149.0	100	34.6
6	4,500	3.8*	150	4.0*
7	9,000	98.1	150	4.6*
8	2,250	10.9*	100	11.6*
9	9,000	11.6*	150	20.7

\* Low ferritin group.

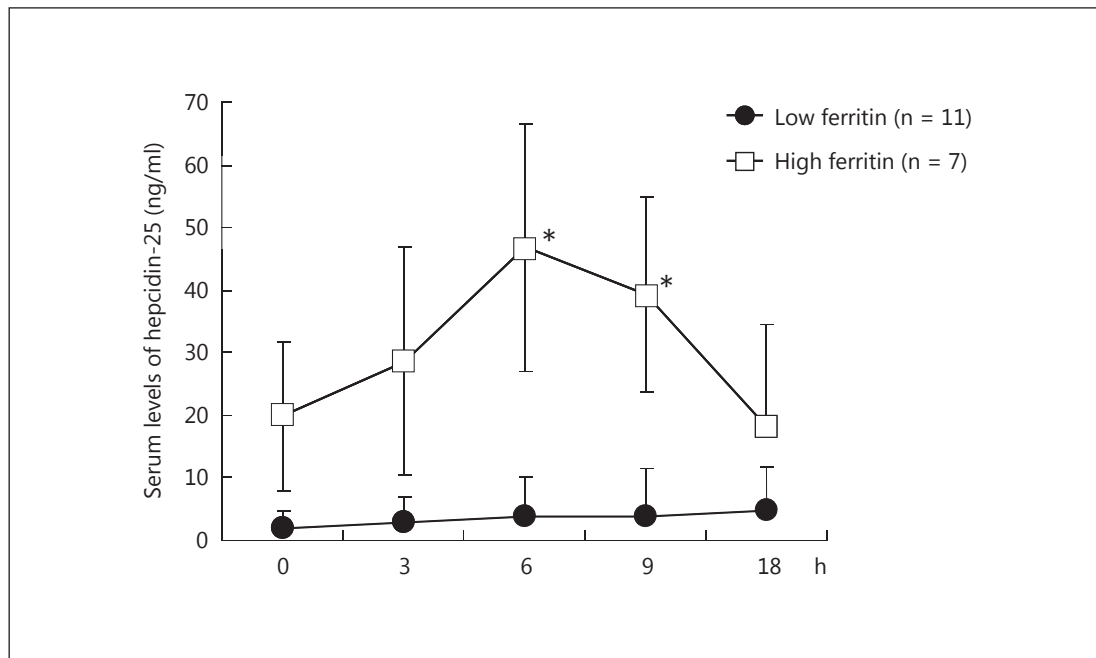
**Table 3.** Effect of rhEPO on anemia and iron parameters in HD patients with low and high serum levels of ferritin

Variable	Low ferritin group		High ferritin group	
	before hepcidin-25 measurement	6 months after rhEPO	before hepcidin-25 measurement	6 months after rhEPO
Hb, g/dl	10.6±2.6	11.1±1.5	11.2±0.7	9.7±0.9*
Ferritin, ng/ml	8.7±3.7	16.8±16.5	55.3±49.4	29.3±21.8*
Transferrin, mg/dl	276.4±25.8	251.4±32.6	232.0±52.5	248.4±58.3

\* p < 0.05 vs. data before the measurement of hepcidin-25.

Based on the baseline levels of serum ferritin before the measurement of hepcidin in two independent studies, the patients were combined and grouped into a low ferritin group (11 patients with serum ferritin levels of <15.0 ng/ml; 5 and 6 patients in the study treated with EPO and PEG-EPO, respectively) and a high ferritin group (7 patients with serum ferritin levels of ≥15.0 ng/ml; 4 and 3 patients in the study with EPO and PEG-EPO, respectively) (table 2). There was no difference in gender ratio between the groups. In the low ferritin group, serum levels of hepcidin-25 were low (<5.0 ng/ml) at all time points of the measurements after rhEPO administration (fig. 2). In contrast, they rose from the baseline of 19.8 ± 12.0 to 46.8 ± 19.8 ng/ml (p < 0.01) at 6 h and 39.3 ± 15.5 ng/ml (p < 0.01) at 9 h after rhEPO administration in the high ferritin group.

To examine the effect of rhEPO on anemia, Hb and iron parameters such as ferritin and transferrin before and 6 months after the measurement of hepcidin were compared between the low and high ferritin groups (table 3). The levels of Hb before the measurement of hepcidin were similar between the groups. In the low ferritin group, the levels of Hb tended to increase from the baseline of 10.6 ± 2.6 to 11.1 ± 1.5 g/dl at 6 months after the measurement of hepcidin, although this did not reach statistical significance. In contrast, they were significantly decreased from the baseline of 11.2 ± 0.7 to 9.7 ± 0.9 g/dl (p < 0.05) in the high ferritin group.



**Fig. 2.** Early response of serum hepcidin-25 to the intravenous administration of EPO and PEG-EPO in HD patients with low and high serum levels of ferritin. Based on the baseline of serum ferritin before the measurement of hepcidin-25 in two independent studies with either EPO or PEG-EPO, the patients were combined and grouped into a low ferritin group (11 patients with serum ferritin levels of <15.0 ng/ml; 5 and 6 patients in the study with EPO and PEG-EPO, respectively) and a high ferritin group (7 patients with serum ferritin levels of  $\geq 15.0$  ng/ml; 4 and 3 patients in the study with EPO and PEG-EPO, respectively). Serum levels of hepcidin-25 were low at all time points of the measurements in the low ferritin group, whereas they rose at 6 and 9 h after intravenous administration of rhEPO in the high ferritin group. \*  $p < 0.01$ , 0 vs. 6 and 9 h.

In the low ferritin group, there was a trend toward an increase in serum levels of ferritin from the baseline of  $8.7 \pm 3.7$  to  $16.8 \pm 16.5$  ng/dl ( $p = 0.06$ ) at 6 months after the measurement of hepcidin, although this did not reach statistical significance. In contrast, they were significantly decreased from the baseline of  $55.3 \pm 49.4$  to  $29.3 \pm 21.8$  ng/dl ( $p < 0.05$ ) at 6 months after the measurement of hepcidin in the high ferritin group. However, serum levels of transferrin before and 6 months after the measurement of hepcidin were similar between the groups.

To examine the influence of inflammation on hepcidin, the values for CRP before and 6 months after the measurement of hepcidin were compared between the low and high ferritin groups. Serum levels of CRP before ( $0.11 \pm 0.16$  vs.  $0.04 \pm 0.04$  mg/dl in the low and high ferritin groups) and 6 months after the measurement of hepcidin ( $0.08 \pm 0.07$  vs.  $0.05 \pm 0.03$  mg/dl in the low and high ferritin groups) did not differ between the groups.

## Discussion

Regarding the late effect of rhEPO on hepcidin or prohepcidin in HD patients, serum levels of hepcidin have been shown to be decreased after rhEPO administration [10, 11, 13]. Similarly, serum prohepcidin levels were decreased after rhEPO administration and accompanied by an increase in the levels of Hb and hematocrit [10]. Serum levels of hepcidin were



negatively correlated with the dose of rhEPO in HD patients, suggesting that the long-term administration of erythropoietin suppresses hepcidin levels [5].

Concerning the early effect of rhEPO on hepcidin, several studies using healthy volunteers have shown an early rise in hepcidin [14, 15] followed by its suppression within 2–3 days after rhEPO administration [15, 16]. Our study showed a similar pattern, since the short- and long-acting rhEPO induced an early and significant rise in serum hepcidin-25 in HD patients. However, the time to reach the maximum levels of hepcidin and return to the baseline after rhEPO was longer than in healthy volunteers [14] probably because of the reduced clearance of hepcidin and rhEPO in HD patients [21]. In our series, an increase in serum hepcidin-25 in response to rhEPO appears to be dependent on ferritin but not on gender or circadian rhythm [5, 20]. In a state of low ferritin, indicative of iron deletion, low hepcidin facilitates iron availability for erythropoiesis by increasing iron absorption and efflux [1, 2]. In support of our finding, poor response of hepcidin to rhEPO has been associated with HD patients with low serum ferritin [22]. A recent *in vitro* study has shown that a low dose of rhEPO increases the hepcidin expression, whereas a high dose of rhEPO suppresses the hepcidin expression in human hepatocytes, depending on the activation of the C/EBP $\alpha$  pathway [23]. In addition, an *in vivo* study has shown that ferritin upregulates the expression of hepcidin in mice [24]. Our data, together with previous data, suggest that, in HD patients, the rhEPO-induced early rise in hepcidin-25 may be dependent on ferritin levels and/or other pathways.

On the contrary, our study showed that serum levels of hepcidin-25 were significantly decreased on days 5–7 after PEG-EPO administration compared to the baseline, suggesting that as the late effect, rhEPO suppresses hepcidin in HD patients, which is similar in healthy volunteers [15, 16]. Although the precise mechanisms are unknown, the rhEPO-induced suppression of hepcidin at a late phase may be mediated by circulating factors or signals derived from bone marrow cells [15, 25, 26] and indirect mechanisms affecting the hemopoietic activity in bone marrow cells [27].

It remains inconclusive whether an alteration of serum hepcidin-25 and ferritin is predictive of the response to rhEPO in HD patients. For example, no difference was found in the serum levels of prohepcidin and hepcidin between HD patients hyporesponsive and responsive to rhEPO [12, 28, 29]. Serum hepcidin was correlated positively with ferritin [5, 29] and negatively with the dose of rhEPO [5], but failed to predict the response to rhEPO. However, lower serum levels of hepcidin [30] and ferritin [21, 31] have been associated with increased resistance to rhEPO in HD patients. In contrast, our study showed a trend toward an increase of Hb after rhEPO in HD patients with low serum levels of hepcidin-25 and ferritin. Furthermore, it was found that the levels of Hb were significantly decreased in those with high serum levels of hepcidin-25 and ferritin. These data suggest that an alteration of serum levels of hepcidin-25 and ferritin may be predictive of the response to rhEPO in HD patients. In support of our finding, serum levels of hepcidin or prohepcidin after rhEPO administration were negatively correlated with Hb in HD patients [10]. Low serum levels of prohepcidin have been associated with a lower dose requirement of rhEPO in HD patients [32]. In addition, increased serum levels of hepcidin or prohepcidin have been associated with rhEPO-resistant HD patients [12, 26]. Our data, together with these previous data, suggest that an alteration of serum levels of hepcidin-25 and ferritin may be predictive of the response to rhEPO in HD patients. Inflammation increases serum levels of ferritin and hepcidin in HD patients [1, 2, 8], which is related to a hyporesponsiveness to rhEPO [28]. Since our patients had normal serum levels of CRP and IL-6, absence of inflammation may be crucial for a better response to rhEPO in HD patients.

In summary, our study showed that in HD patients, the short- and long-acting rhEPOs exert similar biphasic effects on hepcidin-25 in early upregulation followed by late down-

regulation. An early rise in serum hepcidin-25 in response to rhEPO may be dependent on serum ferritin and predictive of the response to rhEPO in HD patients. Further studies using larger numbers of HD patients are necessary to confirm this finding.

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### Disclosure Statement

The authors have no conflicts of interest to disclose.

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