Intra-individual variability of serum hepcidin-25 in haemodialysis patients using mass spectrometry and ELISA

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Abstract

Background. Measurement of serum hepcidin levels may provide a useful alternative to the current methods of determining iron status in chronic haemodialysis (HD) patients. However, the biological variability of this pivotal regulator of iron homeostasis is unclear, and the impact of inflammation, dialysis clearance and iron therapy on hepcidin variability has not been established.

Methods. Two independent studies in chronic HD patients were conducted; serum hepcidin levels were measured at the start of dialysis sessions in 20 UK patients and in 43 Dutch patients by mass spectrometry (MS). Samples from UK patients were also analysed by a competitive enzyme-linked immunosorbent assay (cELISA). Coefficient of variance (CV_1) was calculated and potential factors affecting CV_1 were also examined.

Results. The median CV_1 (inter-quartile range) was 23% (17–28) for the UK MS, 26% (17–48) for the Dutch MS and 23% (17–39) for the UK cELISA. The CV_1 was similar in those patients receiving and those not receiving regular intravenous iron. The CV_1 was not associated with the degree of inflammation. Hepcidin levels were higher following an inter-dialytic period of 3 versus 2 days (P = 0.02).

Conclusions. These findings suggest considerable variability of serum hepcidin levels in HD patients. Inflammation and the use of iron did not impact on the degree of variability, and hepcidin levels were higher after an inter-dialytic period of 3 versus 2 days. These findings need to be taken into account in future studies assessing the utility of serum hepcidin as a guide to the use of iron or erythropoiesis-stimulating agents therapy.

Keywords: anaemia; chronic kidney disease; haemodialysis; hepcidin; variability

Introduction

Hepcidin is a recently discovered small defensin-like peptide that regulates iron metabolism. Hepcidin degrades the cellular iron exporter ferroportin, which is expressed by enterocytes and macrophages, thereby decreasing intestinal iron uptake and causing iron sequestration in the reticulo-endothelial system [1–3]. Hepcidin synthesis is up-regulated in the presence of inflammation or iron overload [4–6] and is reduced in the presence of anaemia [7, 8], hypoxia [4] and iron deficiency [9]. Thus, inflammation decreases the availability of circulating iron, whereas hypoxia or anaemia increases iron release and absorption.

Chronic haemodialysis (HD) patients have been shown to have elevated hepcidin levels [10–17]. This may partly explain why such patients have reduced erythropoiesis despite treatment with erythropoiesis-stimulating agents (ESA) and a seemingly adequate iron status. Traditional markers of iron status are inaccurate for the detection of iron insufficiency in this population, because patients with normal or high ferritin levels and transferrin saturation (TSAT) still respond to intravenous (IV) iron [18, 19]. Moreover, these conventional parameters do not correlate well with bone marrow iron stores. Iron therapy might be a double-edged sword; although iron is necessary for erythropoiesis, excess iron promotes oxidative stress and affects immune effector function [20, 21].

Hepcidin has several potential advantages over conventional iron parameters such as ferritin in optimizing anaemia treatment in patients with renal insufficiency; (i) it directly reflects iron availability and demands for erythropoiesis, (ii) it integrates the input from both inflammatory and erythropoietic pathways and (iii) it reflects the status of iron homeostasis more accurately than single

parameters such as TSAT and soluble transferrin. Hepcidin could thus become an important tool to predict ESA responsiveness and guide treatment with ESA and iron.

The potential role of serum hepcidin was questioned by the recent observations of Ford *et al.*, who reported considerable intra-individual variability of serum hepcidin levels obtained at weekly intervals in a small number of HD patients [22]. The authors noted a relationship with the inflammatory status. Importantly, hepcidin was measured using an enzyme-linked immunosorbent assay (ELISA), which is known to detect not only hepcidin-25, but also its isoforms hepcidin-20 and 22. These biologically inactive isoforms have been reported to accumulate in patients with end-stage renal disease [11, 23]. Thus, the intra-individual variability reported by Ford *et al.* may partly be attributable to the variability of these smaller isoforms.

To meaningfully interpret (changes in) serum hepcidin-25 levels, it is essential to obtain additional data on the variability of serum hepcidin-25, and not total hepcidin levels in HD patients. We have, therefore, extended these observations using a validated liquid chromatography (LC) mass spectrometry (MS) assay [24], and also compared the results with a commercially available ELISA (Bachem, UK). Mass spectrometric techniques have been utilized for the quantitation of hepcidin [24, 25]. Such MS assays are generally regarded as the 'gold standard' but are more technically demanding. The use of MS circumvents inherent problems in the analysis because it allows the absolute levels of hepcidin-25 to be assessed.

Our aim was to determine the intra-individual variability of serum hepcidin in a cohort of HD patients by measuring the coefficient of variance (CV_1) for hepcidin using different assays. Furthermore, we aimed to identify significant determinants of intra-individual variability. Independent studies were designed and conducted in the UK and the Netherlands. Owing to substantial differences in data collection, fully combining the analysis proved impossible. Therefore, the methods and results of the UK and Dutch study will be discussed separately.

Materials and methods

Part I—UK study

Study design. All patients dialysing on two successive days were invited to participate. Patients were excluded if they demonstrated any signs of acute or occult bleeding, had an acute bacterial infection within 4 weeks, a haematological dyscrasia other than anaemia, acute or chronic liver disease or an active malignancy.

Nine consecutive pre-dialysis blood samples were scheduled over a period of 3 weeks during October 2010 at the Department of Renal Medicine, King's College Hospital, London, UK. Two major factors known to affect serum hepcidin levels were prospectively assessed: the administration of IV iron and the degree of inflammation. High-sensitivity C-reactive protein levels (hsCRP) were measured at each of the nine time-points. A single mid-study sample was taken from each patient for measurement of serum ferritin, at least 1 week after any administration of IV iron. The data were also examined according to the day of the week on which the samples were taken, to detect the influence of the inter-dialytic period. Ten patients were dialysed on Mondays, Wednesdays and Fridays, and 10 were dialysed on Tuesdays, Thursdays and Saturdays. The results obtained on Monday or Tuesday were pooled (Mon/Tue), as these samples were taken after an inter-dialytic period of 3 days. Similarly, samples taken on Wednesday or Thursday (Wed/Thurs) were pooled, as were samples taken on a Friday or Saturday (Fri/Sat).

All patients gave informed consent for participation in the study, which was conducted in accordance with the Declaration of Helsinki, and was approved by the London Research Ethics Committee 1 (LREC 09/H0718/034).

Sample analysis. All samples were drawn from the dialysis catheter or fistula immediately before the start of dialysis. The samples were centrifuged, and aliquots of sera were immediately stored in cryotubes at −80° Celsius. Samples were then transferred to two separate laboratories (King's College London, UK, and the University of Bialystok, Poland), for measurement of serum hepcidin levels (LC MS/MS and a commercially available ELISA, respectively). The samples were processed in a single run by two different operators. hsCRP levels were determined using turbidimetry (P.Z. Cormay, Lublin, Poland).

Hepcidin levels were measured by using a high-performance liquid chromatography tandem MS technique, which has an intra-assay CV_1 of 2.3% at hepcidin concentrations of 156 ng/mL and 4.0% at concentrations of 312 ng/mL [24].

The same samples were analysed by ELISA (Bialystok, Poland) using a commercially available kit (Bachem, UK). The reported normal range for this assay is 0.02–25 ng/mL, and both the calculated intra- and inter-assay variations are below 10%.

Statistical analysis. The CV_1 (hepcidin) for every individual was calculated from all nine hepcidin values acquired for each patient. The CV_1 was defined as the standard deviation divided by the mean. The median and mean CV_1 were calculated to determine and compare the variability in serum hepcidin for both assays.

The CV_1 values of those receiving IV iron therapy were compared with those not receiving iron. hsCRP level values were correlated with the corresponding hepcidin values. Hepcidin values obtained after an inter-dialytic period of 3 days were compared with the values after an inter-dialytic period of 2 days.

Data were expressed as mean (±SD) or median (inter-quartile range, IQR) where appropriate. The Shapiro–Wilks test was used to test for normality of distribution. Comparisons between groups were performed using either a paired Student's *t*-test or Wilcoxon rank-sum test for normally and non-normally distributed variables, respectively. Bivariate correlation coefficients (r) were calculated using the Pearson's product formula. A P-value of <0.05 was considered statistically significant.

Analyses were performed using Statistical Package for Social Science version 17.0 for Windows XP (SPSS Inc., Chicago, IL).

Part II—Dutch study

Study design. The objective of the study was to assess intra-individual variability in serum hepcidin-25 concentration over 6 weeks in chronic HD patients and to identify significant determinants. Blood samples were collected once a week during 6 consecutive weeks, before the start of dialysis. Samples were collected from August to October 2010 at the Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands. To obtain unbiased data, we included all patients who agreed to participate. ESA and IV iron administration were continued according to current prescription during the study period.

In accordance with Dutch ethical regulations, all patients consented that blood samples would be used for medical research.

Sample analysis. All samples were drawn from the dialysis catheter or fistula immediately before the start of dialysis, and were processed and stored in polypropylene tubes at −80°C. Routine laboratory parameters and hepcidin levels were measured within 8 h and 6 months of collection, respectively.

Serum hepcidin was measured by a combination of weak cation exchange chromatography and time-of-flight MS as recently described [26]. The intra-run CV_1s is 2.2% at 8.6 ng/mL, 3.7% at 22 ng/mL and 2.3% at 37.4 ng/mL. The inter-run CV_1s is 9.1% at 21.8 ng/mL and 3.9% at 36 ng/mL. Of note, all samples from an individual patient were measured in a single run. The median reference level of serum hepcidin-25 is 11.7 ng/mL, with a range of 1.4–38.8 ng/mL [27]. All other laboratory parameters were measured using standard automated techniques.

Statistical analysis. Intra-individual variability in serum hepcidin and other variables was expressed as the CV_1 (the standard deviation divided by the mean). Week-to-week CV_1s were calculated for each patient, as well as the mean, median and range of CV_1s for serum hepcidin

isoforms, haemoglobin, C-reactive protein (CRP), iron, TSAT and ferritin. Parameters between high and low variability (i.e. above and below median CV_1) groups were compared using the independent t-test for parametric data and the χ^2 test for categorical data.

Correlation coefficients were used to assess associations between laboratory parameters. Correlations were calculated using Pearson's correlation test. If data were skewed, log transformation was used. To determine independent baseline predictors of the intra-individual variability of serum hepcidin, multiple regression analysis was applied. Predictors that showed high collinearity ($r > 0.8$) were not simultaneously included in the analysis. Potential non-linear dose response relationships were checked using fractional polynomials. Robustness of the various models was checked with a Jack-knife re-sampling technique. The unicity of each regression solution was evaluated by interchanging highly correlated baseline predictors. A P-value <0.05 was considered significant for all analyses. Statistical analysis was performed using STATA 10 (Statacorp, TX).

Results

Part I—UK study

Patient demographics and baseline laboratory data are summarized in Table 1. Twenty chronic HD patients (14 males) aged between 35 and 85 years were included. Each patient underwent dialysis three times weekly. Patients had been receiving HD for a mean of 4.1 ± 4.6 years, and all were stable for at least 3 months. All except one patient were dialysed in the afternoon. All except one patient had been on a stable dose of ESA for >4 weeks. Eight patients (40%) had received no IV iron for 4 weeks

Table 1. Demographics and baseline characteristics

	UK study	Dutch study
n (% male)	20 (70)	43 (56)
Age (years)	63 ± 16	62 ± 15
Caucasian (%)	45	98
BMI (kg/m^2)	26.1 ± 4.8	24 ± 3.8
Antibiotic use previous month (%)	0	26
Malignancy in history (%)	5	16
Liver disease (%)	0	9
ESA (%)	95	98
ESA dose (× 1000 units)	6 (4–9)	12 (8–15)
Iron supplementation (%)	60	95
Iron dose (mg/week)	100 (100–150)	50 (25–50)
Laboratory		
Hepcidin 25 (ng/mL)	168.2 (127.9–217.6)	46.7 (28.2–73.4)
Hepcidin 20 (ng/mL)	n/a	13.1 (8.9–19.2)
Ferritin (µg/L)	591.5 (321.9–861.1)	376 (209–503)
Iron (µmol/L)	n/a	55.9 (39.1–72.6)
Transferrin saturation (%)	n/a	19.5 (16.7–30.2)
CRP (mg/L)	5.4 (0.8–18.9)	5 (5–24)
Hb (g/dL)	10.9 (9.8–11.8)	11.4 (10.5–11.9)
CV_1		
Hepcidin-25 (%)	23 (17–28)	26 (17–48)
Hepcidin-20 (%)	n/a	13 (9–21)
Ferritin (%)	n/a	12 (11–26)
Iron (%)	n/a	19 (14–30)
Transferrin saturation (%)	n/a	20 (13–29)
CRP (%)	43.2 (26.9–58.1)	41 (8–63)
Hb (%)	n/a	4 (3–5)

Data are presented as mean ± SD and median (inter-quartile range).
To convert hepcidin-25 from ng/mL to nmol/L divide by 2.789.
BMI, body mass index; ESA, erythropoietin stimulating agents; CRP, C-reactive protein; Hb, haemoglobin; CV_1, coefficient of variance.

prior to commencement of the study, and this was maintained for the 3-week study period.

Intra-individual variability. Both assays revealed a considerable degree of biological variability in this patient cohort (Figure 1). The median CV_1 for the MS assay was 23% (IQR 17–28), and for the ELISA was 23% (IQR 17–39) (P = 0.12). A CV_1 of <20% was seen in only 40% of the patients for both assays, a CV_1 of 20–25% was found in 15% for both assays, a CV_1 of 25–30% was seen in 25% (MS) and 10% (ELISA), and a CV_1 of >30% was seen in 20% (MS) and 35% (ELISA).

Inter-method difference in hepcidin values. The MS assay used in this study previously reported serum hepcidin levels of 4.6 ± 2.7 nmoL/L (12.8 ± 7.5 ng/mL) in normal healthy individuals [24]. In the cohort of HD patients recruited to the present study, hepcidin levels were considerably greater [mean 172 ± 70 ng/mL; median of 168 ng/mL (IQR128–218)]. The data for the Bachem ELISA were similar [mean 230 ± 557; median 170 ng/mL (IQR 107–239 ng/mL), P = 0.03].

Effect of intravenous iron administration on CV_1 and hepcidin levels. The CV_1 was not affected by the administration of IV iron (Table 2). The median CV_1 (MS) for hepcidin in the eight patients who did not receive iron therapy was 26% (IQR 19–28) compared with 23% (IQR 2–32) in those who were receiving regular IV iron (P = 0.77). For individual hepcidin profiles for these eight patients, see Supplementary Figure A. Similar results
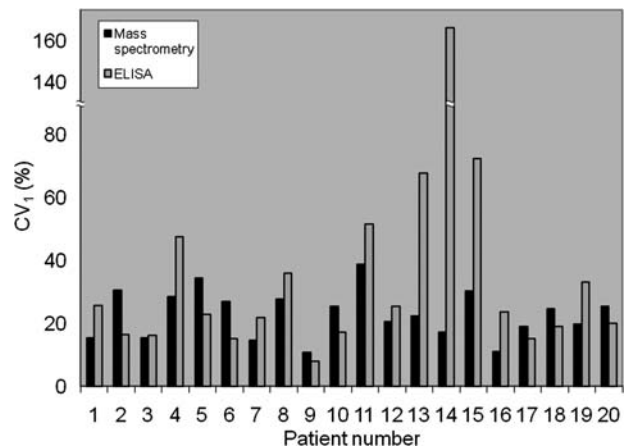

Fig. 1. The coefficient of variance (CV_1) of serum hepcidin-25 in 20 UK patients, using MS and ELISA.

Table 2. Effect of intravenous iron on the variability of serum hepcidin levels, UK study

	Patients receiving IV iron	Patients not receiving IV iron	P-value
n (%)	12 (60)	8 (40)	
CV_1 (MS)	23% (20–32)	26% (19–28)	0.77
CV_1 (ELISA)	23% (17–57)	23% (15–34)	0.29

Data are presented as median (inter-quartile range).

were found using the ELISA assay: median CV_1 for the group not receiving iron was 23% (IQR 15–34) versus 23% (IQR 17–57) for the IV iron group (P = 0.29).

Of the 12 patients receiving IV iron supplementation, 11 were receiving iron administered on a weekly basis across HD. This provided an opportunity to compare hepcidin levels across three time-points: immediately before administration of IV iron, and immediately prior to the next two dialysis sessions. There was a small (nonsignificant) increase in serum hepcidin in the sample taken before the next dialysis session, but overall there was no clear impact of IV iron on serum hepcidin, possibly due to the fairly modest dose administered (100 mg) and the high background levels of serum hepcidin. One patient received IV iron during every dialysis session, but his hepcidin CV_1 values were similar to those of the other patients: 25.6% (MS) and 20.0% (ELISA).

Effect of inflammatory status on CV_1 and hepcidin levels. hsCRP levels were determined in all samples: median 6.1 (IQR 1.1–18.9) mg/L. The correlation between hepcidin (MS) and hsCRP levels in this analysis was weak ($r = 0.15$; P = 0.04) (Figure 2). Mean serum hepcidin levels were significantly increased in the highest hsCRP tertile when compared with the lowest hsCRP tertile [190.4 ± 91.1 versus 161.1 ± 50.7 ng/mL, respectively (P = 0.04)]. We found no correlation between the CV_1 for hsCRP and the CV_1 for hepcidin (MS: $r = 0.38$, P = 0.10; ELISA: $r = -0.18$, P = 0.44).

Effect of inter-dialytic interval on serum hepcidin. Hepcidin levels were higher following a 3-day inter-dialytic interval compared with a 2-day inter-dialytic interval (ANOVA; P = 0.02) (Figure 3). Both assays demonstrated the highest median hepcidin levels on the first day after the weekend [182.8 (138.8–235.0) and 184.1 (111.9–267.7) ng/mL] with MS and ELISA, respectively.

No relationship was found between serum hepcidin and dialysis quantity, haemoglobin, erythropoietin dosage or serum ferritin levels (data not shown).
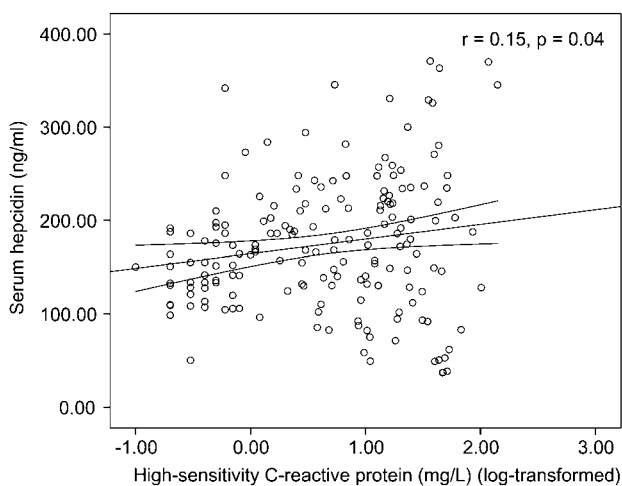
Fig. 2. Correlation between hepcidin (MS assay) and high-sensitivity CRP (log transformed) in UK patients.
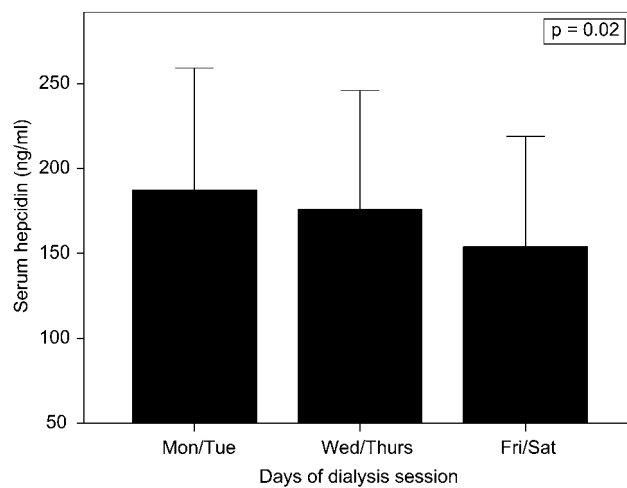
Fig. 3. Mean ($\pm$SD) serum hepcidin-25 levels (MS assay) measured before nine consecutive dialysis sessions, and categorized by the day of the dialysis session in UK patients. Ten patients were dialysed on Mon/Wed/Fri and 10 dialysed on Tues/Thurs/Sat (P = 0.02; ANOVA).
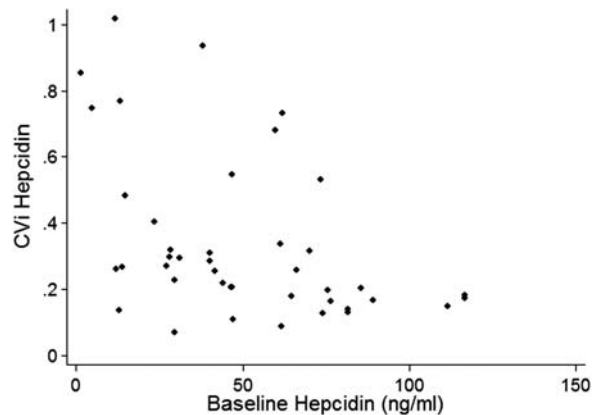
Fig. 4. Correlation of coefficient of variance (CV_1) with baseline hepcidin-25 levels in Dutch patients.

Part II—Dutch study

Patient demographics and baseline laboratory data are summarized in Table 1. We included 43 consecutive chronic HD patients (24 males), aged between 24 and 83 years. Thirty-six patients received HD three times a week for ~4 h, 22 patients were dialysed in the morning, whereas 14 patients were dialysed in the afternoon. Seven patients were treated with nocturnal dialysis four times a week for ~8 h. The median time on dialysis was 2.3 years. The majority of patients were treated with IV iron sucrose ($n = 41$) and epoetin beta ($n = 42$). Iron sucrose was given once weekly, and blood samples were drawn at the start of the session during which IV iron was administered.

Intra-individual variability. One patient died during the study; for this patient only five samples were available for analysis. For the remaining 42 subjects, all six samples were collected according to the study protocol. The median intra-individual coefficient of variation (CV_1) of hepcidin-25 levels over 6 weeks was 26% (IQR 17–48).
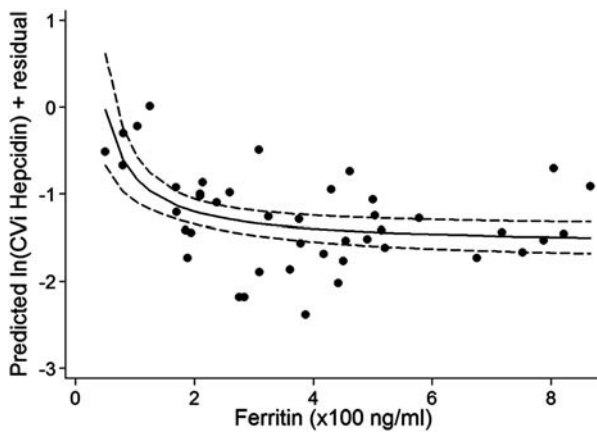
Fig. 5. Corelation between baseline ferritin and intra-individual coefficient of variance (CV_1) of serum hepcidin-25 in Dutch patients.

CV_1 was higher in patients with low levels of serum hepcidin (Figure 4) and serum ferritin (Figure 5). Median CV_1s of ferritin and CRP levels were 12% (IQR 11–26) and 41% (IQR 8–63), respectively.

Determinants of intra-individual variability. Comparison of patients with intra-individual variability of hepcidin-25 above (high variability) and below (low variability) the median CV_1 showed that patients with high intra-individual variability had significantly lower baseline hepcidin and ferritin levels and lower TSAT (Table 3). CRP levels were not significantly different between these two groups. By multivariate regression analysis, we found baseline ferritin and CV_1 of TSAT, but not CRP, to be independent predictors of intra-individual hepcidin-25 variability ($R^2 = 0.54$). Because of collinearity, baseline ferritin can be substituted by baseline hepcidin and CV_1 of TSAT by CV_1 of iron. In the majority of patients, there was no correlation between hepcidin-25 and ferritin, iron, TSAT, CRP or haemoglobin (Figure 6). In the individual patient, change in hepcidin-25 cannot be predicted from change in ferritin or CRP (data not shown).

Limiting the analysis to patients with normal CRP levels did not decrease CV_1 in serum hepcidin-25 levels: median CV_1 22% (IQR 16–34). Likewise, repeating the analysis after exclusion of patients with hepatitis C ($n = 2$), prostate carcinoma ($n = 1$), blood loss or receiving blood transfusion in the month before or during the study ($n = 9$), or patients who received an antibiotic in this period ($n = 8$) did not alter the results [median CV_1 of serum hepcidin-25 levels 27% (IQR 17–48)].

Hepcidin-25 variability was also not dependent on the time of dialysis, as illustrated by a median CV_1 of serum hepcidin-25 levels of 34% (IQR 17–75) in patients on nocturnal dialysis compared with a median CV_1 of 26% (IQR 17–31) in patients on diurnal dialysis (P = 0.40).

Discussion

The results of these studies suggest that there is significant intra-individual variability of serum hepcidin in chronic HD patients, which is consistent with a previous report by

Table 3. Baseline characteristics of Dutch HD patients categorized according to low (below median CV_1) or high (above median CV_1) intra-individual variability of hepcidin-25 levels

	High variability	Low variability	P-value
n (% male)	21 (38%)	21 (48%)	0.35
Age (years)	60 (49–70)	64 (55–74)	0.39
BMI (kg/m²)	24 (22–26)	25 (21–28)	0.36
Antibiotic use previous month (%)	29	24	0.73
Malignancy in history (%)	0	33	0.004
Liver disease (%)	10	10	1.0
ESA (%)	100	95	0.31
ESA dose (× 1000 units)	12 (8–15)	8 (6–15)	0.46
ESA Dose changed (%)	33	38	0.75
Iron suppletion (%)	100	90	0.15
Iron dose (mg/week)	50 (25–50)	50 (25–50)	0.89
Interval between supplementation and hepcidin measurement (weeks)	2 (2–4)	2 (2–4)	0.52
Laboratory			
Hepcidin 25 (ng/mL)	28.8 (13.9–46.9)	64.7 (46.6–81.7)	0.0002
Hepcidin 20 (ng/mL)	4.0 (2.7–5.1)	11.2 (7.5–14.2)	0.03
Ferritin (ng/mL)	237 (170–417)	454 (376–520)	0.008
Iron (µmol/L)	44.7 (33.5–61.5)	61.5 (39.1–89.4)	0.07
Transferrin saturation (%)	18.2 (13.8–21.7)	24.4 (18.4–34.5)	0.01
CRP (mg/L)	5 (5–14)	5 (5–25)	0.73
Hb (g/dL)	11.4 (10.3–11.8)	11.3 (10.8–11.9)	0.31
CV_1			
Hepcidin-25 (%)	37 (32–70)	16 (13–20)	–
Hepcidin-20 (%)	15 (9–26)	11 (8–14)	0.02
Ferritin (%)	21 (12–29)	11 (8–13)	0.03
Iron (%)	26 (15–42)	16 (14 –22)	0.002
Transferrin saturation (%)	27 (15–45)	17 (13–22)	0.004
CRP (%)	59 (15–70)	39 (0–46)	0.09
Hb (%)	4 (3–7)	3 (3–5)	0.13

Data are presented as mean ± SD and median (inter-quartile range), percentages are compared with χ^2-test and continuous variables with t-test.
To convert hepcidin-25 from ng/mL to nmol/L divide by 2.789.
BMI, body mass index; ESA, erythropoietin-stimulating agents; CRP, C-reactive protein; Hb, haemoglobin; CV_1, coefficient of variance.

Ford *et al.* [22]. The study by Ford *et al* had several limitations; the authors studied a small number ($n = 28$) of predominantly African-American dialysis patients, and they did not report the timing of the blood sample and the duration of the inter-dialytic interval. However, the most important limitation of this study by Ford *et al.* was the use of an ELISA assay to measure hepcidin. This is known to cross-react with hepcidin isoforms, and the contribution of this lack of specificity to their findings of hepcidin variability was impossible to ascertain. In the current studies, however, we utilized specific MS assays for hepcidin-25, and confirmed significant hepcidin variability. We also attempted to examine factors that could potentially influence hepcidin levels (IV iron and the degree of inflammation).

The hepcidin results obtained using MS were generally lower than those obtained using the ELISA. This is likely to be due to the fact that the ELISA is detecting the smaller isomers of hepcidin as well as hepcidin-25.
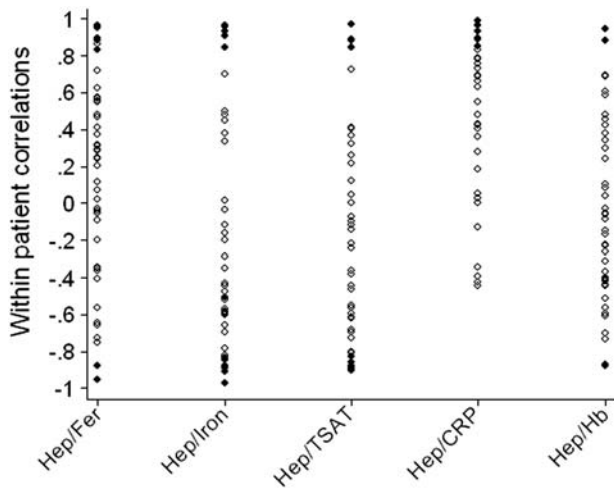
Fig. 6. Distribution of intra-individual correlation between hepcidin-25 and ferritin, iron, TSAT, CRP and haemoglobin in Dutch patients. Squares represent intra-individual correlations (*r*-values) between hepcidin and different variables. Open squares: P > 0.05. Solid squares: P ≤ 0.05.

Nevertheless, as in previous studies, hepcidin levels in HD patients were consistently elevated compared with healthy individuals. Both assays showed a similar degree of hepcidin variability, and the median CV_1 values were almost identical for MS versus ELISA.

Higher hepcidin levels (MS) were seen following a 3-day inter-dialytic interval versus a 2-day interval, possibly due to greater generation of hepcidin during the longer dialysis free period.

It has previously been demonstrated that inflammation (acute and chronic), IV iron and erythropoietin administration [12] influence hepcidin levels. In our parallel studies, we were unable to demonstrate a convincing association between CRP and hepcidin levels, in contrast to the findings of Ford *et al.* and the multivariate analyses by Zaritsky *et al.* [16]. Interestingly, when using a different MS assay and a different study design, very similar CV_1 values (26%) were observed in the Dutch cohort to those seen in the UK study (24%).

We previously suggested that measurement of serum hepcidin might have a role in predicting CKD patients' response to IV iron therapy [28]. This was investigated by Tessitore *et al.* in 56 HD patients [29]. Serum hepcidin did not predict the response to IV iron loading. In addition, it was demonstrated that mean serum hepcidin levels did not change following IV iron loading, confirming our findings and also those of Weiss *et al.* [13]. It is possible, therefore, that these negative results may be partly due to significant hepcidin variability.

It is presently unclear whether the variability is related to specific, hitherto unexplained features that are characteristic of the dialysis procedure. Future studies must evaluate CV_1 in pre-dialysis patients to answer this question. The large and unexplained variability suggests that it may be difficult to use serum hepcidin-25 levels to guide treatment of anaemia in the individual patient. However, we caution against premature conclusions. Measurements

of serum hepcidin-25 in blood samples collected at shorter time intervals, and with and without administration of ESA and IV iron are needed to assess the role of serum hepcidin-25 in HD patients.

In conclusion, we have independently demonstrated that variability in serum levels of hepcidin-25 in HD patients exceeds 20%, even when using an MS assay. No major impact of IV iron or inflammatory status was observed, although a minor effect of length of inter-dialytic interval was seen. These data have implications for studies examining factors affecting hepcidin levels, and suggest that the utility of serum hepcidin as a marker of iron status in HD patients is limited.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

Conflict of interest statement. None declared.

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Circulating cell-free DNA in hemodialysis patients predicts mortality

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Abstract

Background. Circulating cell-free DNA (CFD) appears following cell damage and DNA release, and increases in hemodialysis (HD) patients particularly following HD. We hypothesized that CFD is an integrative marker of tissue damage and can be an independent predictor for all-cause mortality in HD patients.

Methods. In a prospective study, CFD levels before and after HD were evaluated in 31 chronic HD patients with no acute disease, using the reported rapid non-cumbersome inexpensive fluorometric assay developed in our laboratory. Follow-up levels were assessed at 18 months in 22 patients. All-cause mortality was a primary endpoint.

Results. During 42 months of follow-up, 13 of the 31 (41.9%) patients died. The decedents were older than the survivors (mean age 69.9 versus 61.5 years, P = 0.06), but did not differ in end-stage renal disease (ESRD) duration, gender, albumin and hemoglobin, diabetes mellitus and weight. Post-dialysis CFD levels were significantly lower in survivors (median 688 versus 880 ng/mL, P = 0.01). The sensitivity and specificity of CFD levels of 850 ng/mL to predict 42 months (3.5 years) mortality were 73 and 75%, respectively, and the area under the receiver-operating characteristic curve was 0.77 [95% confidence interval (CI) 0.60–0.94]. The Cox proportional hazard regression model showed that CFD higher than 850 ng/mL adjusted for age,